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(71) Applicant:

Mixson, Archibald James
Rockville, MD 21201 (US)

(72) Inventor:

Mixson, Archibald James
Rockville, MD 21201 (US)

(74) Representative:

Sieckmann, Ralf, Dr. et al
Cohausz Hase Dawidowicz & Partner,
Patent- und Rechtsanwaltskanzlei,
Schumannstrasse 97
40237 Düsseldorf (DE)

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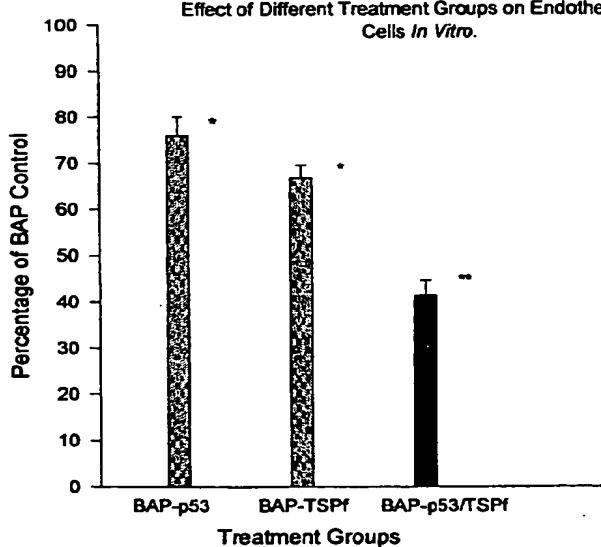
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(54) Cationic vehicle: DNA complexes and their use in gene therapy

(57) Cationic vehicles:DNA complexes comprising DNA encoding an anti-angiogenic peptide or DNA encoding a tumor suppressor protein and DNA encod-

ing an anti-angiogenic peptide, as well as their use in gene therapy, are disclosed.

Figure 2
Effect of Different Treatment Groups on Endothelial Cells *In Vitro*.



-- BAP vs BAP-p53 or BAP-TSPf, p<0.05

-- BAP-p53 or BAP-TSPf vs BAP-p53/BAP-TSPf, p<0.01

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Description**FIELD OF THE INVENTION**

5 The present invention relates to cationic vehicles DNA complexes (i.e. cationic liposome:DNA complexes, cationic polymer:DNA complexes) comprising DNA encoding an anti-angiogenic peptide, or DNA encoding a tumor suppressor protein and DNA encoding an anti-angiogenic peptide, and their use in gene therapy.

BACKGROUND OF THE INVENTION

10

I. Gene Therapy

Development of gene therapy techniques is approaching clinical realization for the treatment of neoplastic and metabolic diseases. The main obstacle in the treatment of malignant diseases, however, remains in the vector delivery system of the transgene to a distant target tissue.

15 Vectors carrying genes are commonly divided into viral and non-viral vector categories. Unfortunately, all vectors described to date have significant limitations. For example, replication-deficient retroviral vectors can efficiently transfect dividing cells. Local intratumoral injection of retroviruses that contain a thymidine kinase transgene has been used successfully to affect regression of gliomas (Culver et al, *Science*, 256:1550-1552 (1992)). However, retroviruses have 20 the potential to cause insertional mutagenesis. As a result, their use has been limited to either direct injection of tumors or to *ex vivo* gene transfer trials. Unlike retroviral vectors, adenoviral vectors can also transfect non-dividing cells, and their ability to cause insertional mutagenesis is greatly reduced. However, they have the undesirable potential to activate the immune system in humans (Crystal, *Science*, 270:404-410, (1995)). Attempts are underway to minimize the 25 immunogenicity of the adenoviral vectors, but the potential toxicity of viral vectors will most likely limit their use for systemic delivery of genes in the near future.

Non-viral vectors of DNA include liposomes, peptides, proteins and polymers (Ledley, *Current Opinion in Biotechnology*, 5:626-636 (1994)). Of these, liposomes are the most commonly used non-viral vectors of DNA. The major advantage of liposomes over retroviruses is that DNA is not incorporated into the genome, and unlike adenoviral vectors, they are not immunogenic. However, the major limitation of liposomes is that they are not as efficient as viral 30 vectors in transfecting many cell types. Until recently, their medical utility was limited by their rapid uptake by phagocytic cells. Interest in liposomes as a vector was rejuvenated by two technological advances that have produced a renaissance in the field. First, stearically stabilized (Stealth) liposomes represent a significant breakthrough in that they are non-reactive, and are not readily taken up by the reticuloendothelial system (RES). Stealth liposomes are composed of 35 lipids rich in oxygen in their head group (ethylene glycol or glycolipids) which provide a stearic barrier outside of the membrane. As a result, Stealth liposomes remain in the blood for up to 100 times longer than conventional liposomes, and can thus increase pharmacological efficacy (Papahajopoulos, *In: Stealth liposomes*, Ed., Lasic et al, CRC Press (1995); and Lasic et al, *Science*, 267:1275-76 (1995)). However, Stealth liposomes are still not particularly efficient in transfection of cells or as vectors for DNA.

The second significant advance in liposome technology has been the use of cationic liposomes complexed to negatively-charged DNA. Cationic liposomes can condense DNA, and increase transfection yields several orders of magnitude. In the cationic liposome:DNA complex, the nucleic acids or oligonucleotides are not encapsulated, but are 40 simply complexed with small unilamellar vesicles by electrostatic interactions. The exact nature of the cationic liposome:DNA complex is not clear, but intricate topological rearrangements of the cationic liposome:DNA complex occur, including DNA condensation, liposome aggregation, and fusion. This supramolecular complex can be added to cells *in vitro*, injected parenterally, or aerosolized for pulmonary applications (Lasic et al, *Science*, 267:1275-1276 (1995)). Further, the intravenous injection into mice of high concentrations of the CAT gene (100 µg or greater) complexed with 45 cationic liposomes has been found to result in 40% transfection efficiency of well vascularized tissues, such as the spleen (Zhu et al, *Science*, 261:209-211 (1993)). However, a major challenge of gene therapy remains the systemic delivery 50 of transgenes to the tumor or peritumoral area that will effectively decrease the size of primary tumors and their metastases. This is because unlike the spleen and bone marrow, which are highly vascular and have a high capacity to filter macromolecules from the blood stream, most organs and tumors do not have this capacity, and the transfection efficiency of these tissues with liposomes is low (Marshall, *Science*, 269:1051-1055 (1995)). In addition, another limitation 55 of cationic liposome: DNA complexes is that their 1/2 life in the blood stream is less than one hour (Allen et al, *In: Liposome Technology*-Vol. III, Ed., Gregoriadis G et al, CRC Press (1993)). Sufficient transfection of the target cell by vectors carrying therapeutic genes has thus far been the rate-limiting step in gene therapy.

II. Tumor Suppressor Genes

5 Tumor suppressor genes are well-known in the art, and include the p53 gene (Baker et al, *Science*, 249:912-915 (1990)), the p21 gene (El-Deiry et al, *Cell*, 75:817-825 (1993); and Harper et al, *Cell*, 75:805-816 (1993)), and the rb gene (Bookstein et al, *Science*, 247:712-715 (1990)).

10 Mutations in the tumor suppressor gene p53 are known to occur in over 50% of human tumors, including metastatic breast cancer (Vogelstein, *Nature*, 348:681-682 (1990)). Breast cancer is one of the leading causes of death in women in North America and Western Europe, affecting nearly 10% of this population living to 80 years of age, and one million new cases are predicted by the end of this decade (Miller et al, *Int. J. Cancer*, 37:173-177 (1986)). Although the molecular basis of multistage carcinogenesis in breast cancer is not well understood, the metastatic potential of breast cancers has been correlated with the presence of point mutations in the p53 gene (Wang et al, *Oncogene*, 8:279-288 (1993)). Various groups have found that reintroduction of the wild-type P53 into a tumor cell has the therapeutic potential to inactivate the proliferative effects of the mutated product (Bookstein et al, *Cancer*, 71:1179-1186 (1993); Chen et al, *Science*, 250:1576-1580 (1990); and Baker et al, *Science*, 249:912-915 (1990)). For example, *in vitro* transfection and retroviral-mediated transfer of a single copy of the p53 transgene into a variety of tumor cells, including breast cancer cells, was found to result in a decrease in growth rate and/or attenuated tumor development once those transfected cells were implanted into nude mice (Wang et al, *Oncogene*, 8:279-288 (1993); Baker et al, *Science*, 249:912-915 (1990)); Bookstein et al, *Science*, 247:712-715 (1990); Cheng et al, *Cancer Res.*, 52:222-226 (1992); Isaacs et al, *Cancer Res.*, 51:4716-4720 (1991); Diller et al, *Mol. Cell. Biol.*, 10:5772-5781 (1990); Chen et al, *Oncogene*, 6:1799-1805 (1991); and Zou et al, *Science*, 263:526-529 (1994)). In addition, intratracheal injection of a retrovirus containing the p53 transgene has been shown to significantly inhibit the growth of lung tumors (Fujiwara et al, *J. Natl. Cancer Inst.*, 86:1458-1462 (1994)). Further, systemic intravenous administration of a β -actin promoter-containing vector containing the p53 coding sequence complexed to cationic liposomes has been found to affect the tumor growth of a malignant line of breast cancer cells injected into nude mice (Lesoon-Wood et al, *Proc. Am. Ass. Cancer Res.*, 36:421 (1995); and Lesoon-Wood et al, *Human Gene Ther.*, 6:39-406 (1995)). Of the 15 tumors treated in this study, four of these tumors did not respond to treatment. Because of the unresponsiveness of these tumors, new therapies were sought in the present invention to more effectively decrease the size of these tumors. Based on the *in vitro* data concerning p53, one might expect that p53 decreases the size of the tumors due to efficient transfection of the tumor. However, less than 5% of the tumor was transfected after three injections of a cationic liposome:marker (CAT) gene. Furthermore, some 25 endothelial cells of the tumor were transfected with this marker gene. Thus, the primary target of cationic liposome:p53 complex may be the vasculature system of the tumor. Given that angiogenesis is critical for the development of any human tumor, as well as for metastastases (Fidler et al, *Cell*, 79:185-188 (1994)), this therapy should be widely applicable to a wide variety of tumors.

30 p53 coordinates multiple responses to DNA damage. DNA damage results in an increase in the level of the p53 protein. Following DNA damage, an important function of wild-type p53 function is to control the progression of cells from G1 to S phase. Recently, several groups have found that p53 transcriptionally activates a p21 kd protein (also known as WAF1 or Cip1), an inhibitor of cyclin-dependent kinases (CDKs) (El-Deiry et al, *supra*; and Harper et al, *supra*). Inhibition of CDK activity is thought to block the release of the transcription factor E2F, and related transcription factors from the retinoblastoma protein RB, with consequent failure to activate transcription of genes required for S phase entry (Harper et al, *supra*; and Xiong et al, *Nature*, 366:701-704 (1993)). Evidence consistent with the model that pRb is a downstream effector of p53-induced G1 arrest has recently been reported (Dulic et al, *Cell*, 76:1013-1023 (1994)). Thus, p53 regulates cell cycle through two proteins: p21 and rb.

III. Anti-Angiogenic Proteins

45 Proteins with anti-angiogenic activities are well-known and include: thrombospondin I (Kosfeld et al, *J. Biol. Chem.*, 267:16230-16236 (1993); Tolsma et al, *J. Cell Biol.*, 122:497-511 (1993); and Dameron et al, *Science*, 265:1582-1584 (1995)), IL-12 (Voest et al, *J. Natl. Cancer Inst.*, 87:581-586 (1995)), protamine (Ingber et al, *Nature*, 348:555-557 (1990)), angiostatin (O'Reilly et al, *Cell*, 79:315-328 (1994)), laminin (Sakamoto et al, *Cancer Res.*, 51:903-906 (1991)), and a prolactin fragment (Clapp et al, *Endocrinol.*, 133:1292-1299 (1993)). In addition, several anti-angiogenic peptides have been isolated from these proteins (Maione et al, *Science*, 247:77-79 (1990); Woltering et al, *J. Surg. Res.*, 50:245-251 (1991); and Eijan et al, *Mol. Biother.*, 3:38-40 (1991)).

50 Thrombospondin I (hereinafter "TSPI") is a large trimeric glycoprotein composed of three identical 180 kd subunits (Lahav et al, *Semin. Thromb. Hemostasis*, 13:352-360 (1987)) linked by disulfide bonds (Lawer et al, *J. Cell Biol.*, 103:1635-1648 (1986); and Lahav et al, *Eur. J. Biochem.*, 145:151-156 (1984)). The majority of anti-angiogenic activity is found in the central stalk region of this protein (Tolsma et al, *supra*). There are at least two different structural domains within this central stalk region that inhibit neovascularization (Tolsma et al, *supra*).

55 Besides TSPI, there are five other proteins (fibronectin, laminin, platelet factor-4, angiostatin, and prolactin frag-

ment) in which peptides have been isolated that inhibit angiogenesis. In addition, analogues of the peptide somatostatin are known to inhibit angiogenesis.

5 Fibronectin (FN) is a major surface component of many normal cells, as well as a potent cell spreading factor. During transformation, the loss of cellular FN has been observed. Furthermore, the addition of fibronectin to transformed cells restores the normal phenotype. It has been found that either heparin-binding or cell-adhesion fragments from FN can inhibit experimental metastasis, suggesting that cell surface proteoglycans are important in mediating the adhesion of metastatic tumor cells (McCarthy et al, *J. Natl. Cancer Inst.*, 80:108-116 (1988)). It has also been found that FN and one of its peptides inhibits *in vivo* angiogenesis (Eijan et al, *Mol. Biother.*, 3:38-40 (1991)).

10 Laminin is a major component of the basement membrane, and is known to have several biologically active sites that bind to endothelial and tumor cells. Laminin is a cruciform molecule that is composed of three chains, an A Chain and two B chains. Several sites in laminin have been identified as cell binding domains. These sites promote cellular activities *in vitro*, such as cell spreading, migration, and cell differentiation. Two peptides from two sites of the laminin B1 chain are known to inhibit angiogenesis (Grant et al, *Path. Res. Pract.*, 190:854-863 (1994)).

15 Platelet factor-4 (PF4) is a platelet α -granule protein originally characterized by its high affinity for heparin. The protein is released from platelets during aggregation as a high molecular weight complex of a tetramer of the PF4 polypeptide and chondroitin sulfate, which dissociates at high ionic strength. PF4 has several biological properties including immunosuppression, chemotactic activity for neutrophils and monocytes as well as for fibroblasts, inhibition of bone resorption, and inhibition of angiogenesis. The angiostatic properties of human PF4 are associated with the carboxyl-terminal, heparin binding region of the molecule. A 12 amino acid synthetic peptide derived therefrom has been discovered to have marked angiostatic affects (Maione et al, *Science*, 247:77-79 (1990)).

20 Although somatostatin is not a protein, it is a naturally-occurring cyclic 14 amino acid peptide whose most-recognized function is the inhibition of growth hormone (GH) secretion. Somatostatin is widely distributed in the brain, in which it fulfills a neuromodulatory role, and in several organs of the gastrointestinal tract, where it can act as a paracrine factor or as a true circulating factor. The role played by the neuropeptide somatostatin, also known as somatotropin release inhibitory factor (SRIF), in human cancer is not well understood. Recent investigations involving somatostatin receptors in normal and neoplastic human tissues suggest that the action is complex, and involves both direct and indirect mechanisms. One of the anti-tumor mechanisms of these synthetic somatostatin analogues may be an anti-angiogenic effect (Woltering et al, *J. Surg. Res.*, 50:245-50 (1990)). In a recent study, the ability of native somatostatin and nine somatostatin analogues to inhibit angiogenesis were evaluated. The most potent somatostatin analogue was found to be approximately twice as potent as the naturally-occurring somatostatin (Barrie et al, *J. Surg. Res.*, 55:446-50 (1993)).

25 Angiostatin is a 38 kDa polypeptide fragment of plasminogen. Whereas plasminogen has no fibrinolytic activity, angiostatin has marked angiogenic activity (O'Rielly MS, et al *Cell*, 79:315-28 (1994)). Angiostatin was isolated when it was observed that the primary tumor suppressed metastases. That is, when the primary tumor was removed, the metastases grew. Administration of angiostatin blocks neo-vascularization and growth of metastases.

30 Finally, a 16kd fragment of prolactin has been found to be angiogenic. Similar to plasminogen, prolactin is not anti-angiogenic but the prolactin fragment is a potent *in vivo* and *in vitro* inhibitor of angiogenesis (Clapp C, et al. *Endocrinology*, 133:1292-1299 (1993)).

35 Despite the evidence that anti-angiogenic peptides are effective anti-tumor agents, as well as the great deal of interest in targeting genes toward the vasculature, there have been no published reports on effective *in vivo* gene therapy regimens with established anti-angiogenic DNA sequences.

40 There are several reasons why gene therapy utilizing antiangiogenic genes have not been used or why antiangiogenic peptides are effective and the liposome: antiangiogenic gene may not be. First, there are significant physical differences between the liposome: DNA complexes and their peptides. Cationic liposomes have a 1/2 life of less than one hour (Allen TM and Papahajopoulos D, In: *Liposome Technology*-Vol. III, Ed., Gregoriadis G et al, CRC Press (1993)), whereas the most effective of the antiangiogenic peptides (i.e angiostatin) have a 1/2 life of two days (Folkman J, The John Krantz, Jr Lecture in Pharmacology, UMAB, 4/30/96). Since cationic liposomes form large aggregates when mixed with DNA, the distribution of these complexes is likely to be quite different from the much smaller peptides (need reference). These properties of the liposomes may account for the low transfection efficiency of a tumor. Therefore, it is uncertain as to whether these liposome:DNA complexes will reach their cellular targets.

45 Furthermore, the exact receptor target or mechanisms of these antiangiogenic peptides are unknown (Tolsma et al, *supra*). For example, it is unknown whether these receptor targets are intracellular or extracellular. The anti-angiogenic genes that are complexed to liposomes encode their respective proteins inside the cell, and proteins without secretory sequences remain inside the cell. Thus, it is unclear that a intracellular antiangiogenic peptide derived from a systemically transfected gene will reach its cellular and/or receptor target.

50 The only transfected antiangiogenic gene that has inhibited tumor growth is full length thrombospondin I. In this study (Weinstat-Saslow et al, *Cancer Research* 54, 6504-6511, (1994)) tumor cells that expressed 15 fold higher levels of the thrombospondin I *in vitro* than baseline cells were implanted into the mice. This transfected full length throm-

bospondin I was secreted from the tumor cells to inhibit angiogenesis, and effectively reduced the tumor by 60%. Thus, this study determined that transfection of 100% of the tumor cells with a highly expressed secreted antiangiogenic gene was able to reduce tumor size.

5 SUMMARY OF THE INVENTION

An object of the present invention is to provide cationic vehicles:DNA complexes, such as liposome complexes containing DNA encoding anti-angiogenic peptides or cationic complexes containing DNA encoding an anti-angiogenic peptides.

10 Another object of the present invention is to provide a method of anti-angiogenic gene therapy.

Still another object of the present invention is to provide liposome complexes containing DNA encoding an anti-angiogenic peptide.

Yet another object of the present invention is to provide liposome complexes containing DNA encoding an anti-angiogenic peptide in combination with DNA encoding a tumor suppressor gene.

15 A further object of the present invention is to provide liposome complexes containing DNA encoding concatamers of the same or different anti-angiogenic peptides.

An additional object of the present invention is to provide a method for inhibiting tumor growth in a subject, or alternatively, to use these complexes for the production of a medicament, especially for inhibiting tumor growth in a subject.

These and other objects of the present invention, which will be apparent from the detailed description of the invention provided hereinafter, have been met in one embodiment, by a cationic liposome:DNA complex comprising DNA encoding an anti-angiogenic peptide and DNA encoding a tumor suppressor protein.

Further objects of the present invention were solved by the features defined in the present set of claims, but especially the preferred embodiments defined in claims 2 to 25.

Based on the present invention, it is anticipated that one skilled in the gene therapy could utilize other cationic carriers (polylysine, polyhistidine, polycat57, Superfect, and polyethylimine) complexed with the antiangiogenic genes to inhibit tumors.

DETAILED DESCRIPTION OF THE INVENTION

30 As discussed above, in one embodiment, the above-described objects of the present invention have been met by a non-viral:DNA complex comprising DNA encoding an anti-angiogenic peptide and DNA encoding a tumor suppressor protein.

The particular non-viral carrier (liposomes-neutral or non-cationic, see below), polyethylimine (Fluka), polylysine (Sigma), polyhistidine (Sigma), Superfect (Qiagen), are not critical to the present invention although cationic liposomes

35 are preferable carriers. Examples of cationic lipids which can be employed in the present invention include 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (Avanti, Birmingham, AL), 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine (Avanti, Birmingham, AL), and (2,3-diol-eyloxy)propyl-N,N,N-trimethylammonium chloride (DOTMA) (Syntex Corp., Palo Alto, CA).

The cationic lipids are preferably used in a mixture with dioleoylphosphatidylethanolamine (DOPE) (Avanti, Birmingham, AL). In the present invention, the amount of cationic lipid present in the mixture is generally in the range of from 100% to 40% (w/w), preferably about 50% (w/w); and the amount of DOPE present in the mixture is generally in the range of from 0% to 60% (w/w), preferably about 50% (w/w); and the amount of pegylated lipid (1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[Poly(ethylene glycol) 2000] present in the mixture is generally in the range of from 0% to 10% (w/w), preferably about 1% (w/w).

45 The particular ligand will be dependent on the the tumor/peri tumor targeted. Examples of targets on tumors include Her2 (breast), CEA (colon), ferritin receptor (breast, lung, and ovary), and the tumor vasculature (α v β 3 integrins or tissue factor). Antibodies directed toward Her2, CEA, and the tumor's vasculature will be coupled to 1% of the pegylated lipid hydroxyl group of the pegylated lipid with a water soluble carbodiimide (1-ethyl-3-(3-dimethylamino-propyl) carbodiimide), and purified over a sepharose CL-6B column. Similarly, ligands to the tumor (ferritin) and/or the vasculature (the peptide, RGD) are covalently attached to the hydroxyl the pegylated lipids.

The particular tumor suppressor gene employed is not critical to the present invention. Examples of such tumor suppressor genes include the p53 gene, the p21 gene (El-Deiry et al, *supra*; and Harper, *supra*), and the rb gene (Bookstein et al, *supra*). The p53 gene is the preferred tumor suppressor gene employed in the present invention.

55 The particular anti-angiogenic peptide encoded by the DNA is not critical to the present invention. Examples of said peptides include a fragment of thrombospondin I (TSPf) having the following amino acid sequence (the amino acid sequences that are known to be anti-angiogenic are underlined):

5 MTEENKELANELRRPPLCYHNGVQYRNNEETVDSCTECHCQNSVTICKVSCP
 IMPCSNATVPDGECCPRCWPSDSADDGWSPSEWTSCSTSCGNGIQQRGRSCDS
 LNNRCEGSSVQTRTCHIQECDKRFKQDGGWSHWSPSSCSVTCGDGVITRITLC
 NSPSPQMNGKPCEGEARETKACKDACPINGGWGPWSPWDICSVTCGGVQKRS
 RL (SEQ ID NO:1),

10

which is encoded by the following DNA sequence (nucleotides 1013-1650 of the TSPI gene; the underlined sequences encode the anti-angiogenic peptides; the bold TAA is the stop codon):

15 ATGACTGAAGAGAACAAAGAGTTGGCCAATGAGCTGAGGCGGCCTCCCCT
 ATGCTATCACAAACGGAGTCAGTACAGAAATAACGAGGAATGGACTGTTG
 ATAGCTGCACTGAGTGTCACTGTCAGAACTCAGTTACCATCTGCAAAAG
 20 GTGTCCTGCCCATCATGCCCTGCTCCAATGCCACAGTTCTGATGGAGA
 ATGCTGTCCTCGCTGTTGGCCCAGCGACTCTGCGGACGATGGCTGGTCTC
 CATGGTCCGAGTGGACCTCCTGTTCTACGAGCTGTGGCAATGGAATTCA
 25 CAGCGCGGCCGCTCGATAGCCTAACAAACCGATGTGAGGGCTCCTC
 GGTCCCAGACACGGACCTGCCACATTCAAGGAGTGTGACAAAAGATTAAAC
 AGGATGGGGCTGGAGCCACTGGTCCCCGTGGTCATCTGTTCTGTGACA
 30 TGTGGTGATGGTGTGATCACAAAGGATCCGGCTCTGCAACTCTCCAGCCC
 CCAGATGAATGGGAAACCCTGTGAAGGCGAAGCGCGGGAGACCAAAGCCT
 GCAAGAAAGACGCCCTGCCCATCAATGGAGGCTGGGTCCTGGTCACCA
 35 TGGGACATCTGTTCTGTCACCGTGGAGGAGGGTACAGAAACGTAGTCT
 CTCTAA (SEQ ID NO:2);

40 a concatamer of TSPI having the following amino acid sequence (the intervening sequence is underlined):

45 MTEENKELANELRRPPLCYHNGVQYRNNEETVDSCTECHCQNSVTICKVSCP
 IMPCSNATVPDGECCPRCWPSDSADDGWSPSEWTSCSTSCGNGIQQRGRSCDS
 LNNRCEGSSVQTRTCHIQECDKRFKQDGGWSHWSPSSCSVTCGDGVITRITLC
 NSPSPQMNGKPCEGEARETKACKDACPINGGWGPWSPWDICSVTCGGVQKRS

50

55

5 RLCVH~~SRM~~TEENKE~~LA~~ELRRPPLCYHNGVQYRNNEEWTVDSCTECHCQNSVTI
 CKKVSCPIMPCSNA~~T~~VPDGECCPRCWPSDSADDGWSPWSEWTSCSTSCGNGIQQ
 RGRSCDSLNNRCEGSSVQRTCHIQECDKRFKQDGGWSHSPWSSCSVTCDGV
 ITRITLCNSPSPQMNGKPC~~E~~GEARETKACKDACPINGGWGPWSPWDICSVTCG
 GGVQKRSRL (SEQ ID NO:3),

10 which is encoded by the following DNA sequence (the intervening sequence is underlined):

15 ATGACTGAAGAGAACAAAGAGTTGCCAATGAGCTGAGGCGGCCTCCCC
 GATAGCTGC~~ACT~~GAGTGT~~ACT~~GTCAGAAACTCAGTTACCATCTGCAAAAA
 GGTGT~~CCT~~GCC~~CC~~CATCATGCC~~CT~~GCTCCAATGCCACAGTT~~CCT~~GATGGAG
 AATGCTGTC~~CT~~CGCTGTTGCC~~CC~~AGCGACTCTGCC~~AC~~GATGGCTGGTCT
 CCATGGTCCGAGTGGACCTCCTGTT~~CT~~TACGAGCTGTGGCAATGGAATTCA
 GCAGCGCGGCCGCTCCTGCGATAGCCTCAACAAACCGATGTGAGGGCTCCT
 CGGTCCAGACACGGACCTGCCACATT~~C~~AGGAGTGTGACAAAAGATTAAA
 CAGGATGGTGGCTGGAGCCACTGGT~~CCC~~GTGGT~~C~~ATCTGTT~~C~~TGTGAC
 ATGTGGT~~G~~ATGGTGT~~G~~ATCACAAGGATCCGGCT~~T~~GCAACTCTCCCAGCC
 CCCAGATGAATGGAAACCC~~T~~GTAAGGCGAAGCGC~~GG~~GAGACCAAAGCC
 TGCAAGAAAGACGCC~~T~~GCC~~CC~~CATCAATGGAGGCTGGGT~~C~~TTGGT~~C~~ACC
 ATGGGACATCTGTT~~C~~TGT~~C~~ACCTGTGGAGGAGGGTACAGAAC~~G~~TAGTC
 GTCTCTGCGT~~C~~GACTCTAGAATGACTGAAGAGAACAAAGAGTTGCC~~AA~~
 TGAGCTGAGGCC~~GG~~C~~CC~~CT~~CC~~CTATGCTATCACAACGGAGTT~~C~~AGTACAGAA
 ATAACGAGGAATGGACTGTT~~G~~ATAGCTGC~~A~~CTGAGTGT~~C~~ACTGT~~C~~AGAAC
 TCAGTT~~A~~CCATCTGCAAAAGGTGT~~C~~CTGCC~~CC~~CATCATGCC~~CT~~GCT~~CC~~AA
 TGCCACAGTT~~C~~CTGATGGAGAATGCTG~~C~~CTCG~~C~~TGTTGCC~~CC~~AGCGACT
 CTGCC~~GG~~ACGATGGCTGGT~~C~~CCATGGTCC~~G~~AGTGGACCT~~C~~CTGTT~~C~~TACG
 AGCTGTGGCAATGGAATT~~C~~AGCAGGCC~~GG~~CC~~C~~T~~G~~CGATAGCCT~~CA~~
 CAACCGATGTGAGGGCT~~C~~CTCGGT~~C~~CAGACACGGACCTGCCACATT~~C~~AGG
 AGTGTGACAAAAGATTAAACAGGATGGTGGCTGGAGCCACTGGT~~CCC~~CG
 TGGTCATCTGTT~~C~~TGT~~G~~ACATGTGGT~~G~~ATGGTGT~~G~~ATCACAAGGATCCG
 GCTCTGCAACTCT~~CC~~AG~~CC~~CC~~CC~~AGATGAATGGAAACCC~~T~~GTAAGGCG
 AAGCGCGGGAGACCAAGCCTGCAAGAAAGACGCC~~T~~GCC~~CC~~CATCAATGGA
 GGCTGGGT~~C~~CTGGT~~C~~ACCATGGACATCTGTT~~C~~TGT~~C~~ACCTGTGGAGG
 55 AGGGGTACAGAAC~~G~~TAGTCGT~~C~~TCTAA (SEQ ID NO:4);

laminin peptide having the following amino acid sequence: MYIGSR (SEQ ID NO:5), which is encoded by the following

DNA sequence (the *Sa*/*I* sites are underlined, and the stop codon is in bold):

GTCGACATGTATATTGGTTCTCGTAA**GTCGAC** (SEQ ID NO:6);

a concatamer of the laminin sequence having the following amino acid sequence (the intervening sequences are underlined):

5 **MYIGSRGKSYIGSRGKSYIGSRGKS** (SEQ ID NO:7), which is encoded by the following DNA sequence (the *Sa*/*I* sites are underlined, and the intervening sequences are in bold):

GTCGACATGTATATTGGTTCTCGTGGTAAAAGATA**AGATATTGGTTCTCGTGGTAAAAGATAAGTCGACC** (SEQ ID NO:8);

10 a peptide from platelet factor-4 having the following amino acid sequence:

MLYKKI~~KK~~LLS (SEQ ID NO:9), which is encoded by the following DNA sequence (the *Sa*/*I* sites are underlined):

15 GTCGACATGCTTATAAGAAGATCATCAAGAAGCTTCTTGAGAGTAA**GTCGAC** (SEQ ID NO:10);

a concatamer of the platelet factor-4 peptide having the following amino acid sequence (the intervening sequences are underlined):

MLYKKI~~KK~~LLSGKSLYKKI~~KK~~LLS**GKSLYKKI~~KK~~LLS** (SEQ ID NO:11), which is encoded by the following DNA sequence (the *Sa*/*I* sites are underlined, and the intervening sequences are in bold):

20 GTCGACATGCTTATAAGAAGATCATCAAGAAGCTTCTTGAGAGTGGTAAAAGA
CTTTATAAGAAGATCATCAAGAAGCTTCTTGAGAGTGGTAAAAGACTTATAAG

25 **AAGATCATCAAGAAGCTTCTTGAGAGTGGTAAAAGATAAGTCGAC** (SEQ ID NO:12);

30 somatostatin inhibitor having the following amino acid sequence: MFCYWKVCW (SEQ ID NO:13), which is encoded by the following DNA sequence (the *Sa*/*I* sites are underlined):

GTCGACATGTTCTGTATTGGAAGGGATTGTGGTAAGTCGAC (SEQ ID NO:14);

a concatamer of somatostatin inhibitor having the following amino acid sequence (the intervening sequences are underlined):

35 MFCYWKVCWGKSF**CYWKVCWGKSF**CYWKVCWGK**S** (SEQ ID NO:15), which is encoded by the following DNA sequence (the *Sa*/*I* sites are underlined, and the intervening sequences are in bold):

40 GTCGACATGTCTTGATTGGAAGGGATTGTGGGTAAAAGATTCTGTATTGG
AAGGGATTGTGGGTAAAAGATTCTGTATTGGAAGGGATTGTGGGTAAAAGA
TAAGTCGAC (SEQ ID NO:16);

45 fibronectin inhibitor having the following amino acid sequence: MGRGD (SEQ ID NO:17), which is encoded by the following DNA sequence (the *Sa*/*I* sites are underlined):

GTCGACATGTCTTGCTTGAAGACTTGA**CTTAAGTCGAC** (SEQ ID NO:18);

a concatamer of fibronectin inhibitor having the following amino acid sequence (the intervening sequences are underlined):

50 MGRGDGK**S**GRGDGK**S**GRGDGKS (SEQ ID NO:19), which is encoded by the following DNA sequence (the *Sa*/*I* sites are underlined, and the intervening sequences are in bold):

GTCGACATGGGTCGTGGTGATGGTAAAAGAGGTCGTGGTATGGTAAAAGA
GGTCGTGGTATGGTAAAAGATAAGTCGAC (SEQ ID NO:20);
angiostatin having the following amino acid sequence:
MVYLSECKTGIGNGYRGTMSRTKSGVACQKGATFPHPVNPYSPSTHPNEGLEEN
YCRNPNDNEQGPWCYTTDPDKRYDYCNIPECEEECMYCSGEKYEGKISKTMMSG
LDCQAWDSQSPHAHGYIPAKFPSKNLKMNYCHNPDGEPRPWCFTTDPTKRWEYC
DIPRCTTPPPPSPTYQCLKGRGENYRGTVSVTVSGKTCQRWSEQTPHRHNRTP
ENFPCKNLEENYCRNPDGETAPWCYTTDSQLRWEYCEIPSCESSASPDQSDSSV
PPEEQTpvvQECYQSDGQSYRGTSSTTITGKKCQSEQTPHR
(SEQ ID NO:21),

20 which is encoded by the following DNA sequence (the *Sac*I sites are underlined):

GTCGACATGGTGTATCTGTCAGAATGTAAGACCGGCATCGGCAACGGC
TACAGAGGAACCATGTCCAGGACAAAGAGTGGTGTGCCTGTCAAAAG
TGGGGTGCACGTTCCCCACGTACCCAACTACTCTCCCAGTACACAT
CCCAATGAGGGACTAGAAGAGAACTACTGTAGGAACCCAGACAATGAT
GAACAAGGGCCTGGTGTACACTACAGATCCGGACAAGAGATATGAC
TACTGCAACATTCTGAATGTGAAGAGGAATGCATGTACTGCAGTGGA
GAAAAGTATGAGGGCAAAATCTCCAAGACCATGTCTGGACTTGACTGC
CAGGCCTGGATTCTCAGAGCCCACATGCTCATGGATACATCCCTGCC
AAATTCCAAGCAAGAACCTGAAGATGAATTATTGCCACAACCTGAC
GGGGAGCCAAGGCCCTGGTGTTCACAACAGACCCCCACCAAACGCTGG
GAATACTGTGACATCCCCGCTGCACAACACCCCCGCCACCCAGC
CCAACCTACCAATGTCTGAAAGGAAGAGGTGAAAATTACCGAGGGACC
GTGTCTGTCACCGTGTCTGGAAAACCTGTCAGCGCTGGAGTGAGCAA
ACCCCTCATAGGTGA GTCGAC (SEQ ID NO:22),

a concatamer of angiostatin having the following amino acid sequence (the intervening sequences are underlined):

5

MVYLSECKTGIGNGYRGTMRTKSGVACQKWGATFPHVPNYSPSTHPNEGLE
 ENYCRNPDNDEQGPWCYTTDPDKRYDCNIPECEEECMYCSGEKYEGKISKTMS
 GLDCQAWDSQSPHAHGYIPAKFPSKNLKMNYCHNPDGEPRPWCFTTDPTKRWEY
 CDIPRCTTPPPPSPTYQCLKGRGENYRGTVSVTSGKTCQRWSEQTPHRHNRT
 PENFPCKNLEENYCRNPDGETAPWCYTTDSQLRWEYCEIPSCESSASPDQSDSS
 VPPEEQTPVVQECYQSDGQSYRGTSSTTITGKKCQSEQTPHRGKSMVYLSECKT
 GIGNGYRGTMRTKSGVACQKWGATFPHVPNYSPSTHPNEGLEENYCRNPDNDE
 QGPWCYTTDPDKRYDCNIPECEEECMYCSGEKYEGKISKTMSGLDCQAWDSS
 PHAHGYIPAKFPSKNLKMNYCHNPDGEPRPWCFTTDPTKRWEYCDIPRCTTPPP
 PPSPTYQCLKGRGENYRGTVSVTSGKTCQRWSEQTPHRHNRTPENFPCKNLE
 NYCRNPDGETAPWCYTTDSQLRWEYCEIPSCESSASPDQSDSSVPPEEQTPVVQ
 ECYQSDGQSYRGTSSTTITGKKCQSEQTPHR (SEQ ID No :23),

10

15

20

which is encoded by the following DNA sequence (the *S*al sites are underlined, and the intervening sequences are in bold):

25

30

GTCGACATGGTGTATCTGTCAGAAGTAAGGACCCGGCATCGGCAACGGCTTACA
 GAGGAACCATGTCCAGGACAAAGAGTGGTGTTGCCTGTCAAAAGTGGGGTGC
 CACGGTCCCCACGTACCCAACTACTCTCCCAGTACACATCCAATGAGGGA
 CTAGAAGAGAACTACTGTAGGAACCCAGACAATGATGAACAAGGGCTGGT

35

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5 GCTACACTACAGATCCGGACAAGAGATATGACTACTGCAACATTCCTGAATG
 TGAAGAGGAATGCATGTACTGCAGTGGAGAAAAGTATGAGGGCAAAATCTCC
 AAGACCATGTCTGGACTTGACTGCCAGGCCTGGGATTCTCAGAGCCCACATG
 CTCATGGATACATCCCTGCCAAATTTCCAAGCAAGAACCTGAAGATGAATTA
 TTGCCACAACCTGACGGGGAGCCAAGGCCCTGGTGCTTCACAACAGACCCC
 10 ACCAAAACGCTGGAAACTGTGACATCCCCGCTGCACAACACCCCCGCC
 CACCCAGCCAACCTACCAATGTCTGAAAGGAAGAGGTGAAAATTACCGAGG
 GACCGTGTCTGTCAACGTGTCTGGAAAACCTGTCAGCGCTGGAGTGAGCAA
 15 ACCCCTCATAGGGTAAAAGAATGGTGTATCTGTCAGAATGTAAGACCGGCA
 TCGGCAACGGCTACAGAGGAACCATGTCAGGACAAAGAGTGTTGCCTG
 20 TCAAAAGTGGGTGCCACGTTCCCCACGTACCCAACTACTCTCCAGTACA
 CATCCCAATGAGGGACTAGAAGAGAACTACTGTAGGAACCCAGACAATGATG
 AACAAAGGGCCTGGTGCTACACTACAGATCCGGACAAGAGATATGACTACTG
 CAACATTCTGAATGTGAAGAGGAATGCATGTACTGCAGTGGAGAAAAGTAT
 25 GAGGGCAAAATCTCCAAGACCATGTCAGGACTTGACTGCCAGGCCTGGGATT
 CTCAGAGCCCACATGCTCATGGATAACATCCCTGCCAAATTTCCAAGCAAGAA
 CCTGAAGATGAATTATTGCCACAACCTGACGGGGAGCCAAGGCCCTGGTGCT
 30 TCACAACAGACCCCACCAAACGCTGGAAACTGTGACATCCCCGCTGCACAA
 CACCCCCGCCACCCAGCCAACCTACCAATGTCTGAAAGGAAGAGGTGAAA
 ATTACCGAGGGACC GTCTGTCAACGTGTCTGGAAAACCTGTCAGCGCTGG
 35 GTGAGCAA ACCCCTCATAGGTGA GTCGAC (SEQ ID NO:24);

prolactin having the following amino acid sequence:

40 MLPICPGGAARQCQVTLRDLFDRAVVLSHYIHNLSEMSEFDKRYTHGRGFI
 TKAINSCHTSSLATPEDKEQAQQMNQKDFLSLIVSILRSWNEPLYHLVTEVR
 GMQEAPEAIALSKAVEIEEQTK (SEQ ID NO:25),

45 which is encoded by the following DNA sequence:

 50

55

5 ATGTTGCCCATCTGCCCCGGCGGGGCTGCCGATGCCAGGTGACCCTTCGAG
 ACCTGTTGACCGCGCCGTCGTCCCTGTCCACTACATCCATAACCTCTCCTC
 AGAAATGTTAGCGAATTGATAAACGGTATAACCATGGCCGGGGGTTTCATT
 10 ACCAAGGCCATCAACAGCTGCCACACTTCTCCCTGCCACCCCCGAAGACA
 AGGAGCAAGCCAACAGATGAATCAAAAAGACTTCTGAGCCTGATAGTCAG
 CATATTGCGATCCTGGAATGAGCCTCTGTATCATCTGGTCACGGAAGTACGT
 GGTATGCAAGAAGCCCCGGAGGCTATCCTATCCAAAGCTGTAGAGATTGAGG
 15 AGCAAACCTAA (SEQ ID NO:26);

15

and
 a concatamer of prolactin having the following amino acid sequence (the intervening sequences are underlined):

20 MLPICPGGAARQCQVTLRDLFDRAVVLSHYIHNLSEM**SEFDKRYTHGRGFITK**
 AINSCHTSSLATPED**KEQAQQMNQKDFLSLIVSILRSWNEPLYHLVTEVRGMQE**
 APEAILSKAVEIEEQTK**KGKSM**LPICPGGAARQCQVTLRDLFDRAVVLSHYIHNL
 25 SEM**SEFDKRYTHGRGFITK**AINSCHTSSLATPED**KEQAQQMNQKDFLSLIVSILRSWNEPLYHLVTEVRGMQE**APEAILSKAVEIEEQTK (SEQ ID NO:27),

30 which is encoded by the following DNA sequence (the intervening sequences are in bold):

ATGTTGCCCATCTGCCCCGGCGGGGCTGCCGATGCCAGGTGACCCTTCGAG
 ACCTGTTGACCGCGCCGTCGTCCCTGTCCACTACATCCATAACCTCTCCTC
 35 AGAAATGTTAGCGAATTGATAAACGGTATAACCATGGCCGGGGGTTTCATT
 ACCAAGGCCATCAACAGCTGCCACACTTCTCCCTGCCACCCCCGAAGACA
 AGGAGCAAGCCAACAGATGAATCAAAAAGACTTCTGAGCCTGATAGTCAG
 40 CATATTGCGATCCTGGAATGAGCCTCTGTATCCTGGTCACGGAAGTACGT
 GGTATGCAAGAAGCCCCGGAGGCTATCCTATCCAAAG**CTGTAGAGATTGAGG**
 AGCAAACCG**GTAAAAGAATGTTGCCCATCTGTCGCCGGCTGCCGATGCC**
 45 AGGTGAC**CCCTTCGAGACCTGTTGACCGCGCCGTCCTGTCCC**ACTACATCC
 ATAAC**CTCTCCTCAGAAATGTTAGCGAATTGATAAACGGTATAACCATGGCC**
 GGGGGTT**CATTACCAAGGCCATCAACAGCTGCCACACTTCTCCCTGCCACCC**
 50 CCGAAGACAAGGAGCAAGCCAACAGATGAATCAAAAAGACTTCTGAGCCTGA
 TAGTCAGCATATTGCGATCCTGGAATGAGCCTCTGTATCATCTGGTCACGGAAG
 TACGTGGTATGCAAGAAGCCCCGGAGGCTATCCTATCCAAAG**CTGTAGAGATTG**
 55 AGGAGCAAACCTAA (SEQ ID NO:28)

5 Increase efficacy will occur with concatamers of the anti-angiogenic genes. This will increase the anti-angiogenic dosage level without changing the amount of vector necessary to deliver these genes. Similar to concatamers, a plasmid with two or more promoters, a plasmid with the IRES sequence (internal ribosomal entry site) between two sequences, and an antiangiogenic peptide with a secretory sequence will increase the delivery of genes to the therapeutic target without markedly increasing the DNA concentration. With regards to the concatamers, the concatamers can extend up to approximately 4400 bases in length (the coding region of a large protein), and the number of concatamers possible will depend on the number of bases of a single anti-angiogenic unit.

10 For fibronectin, the range of concatamers would be about 2 to 66. Although, the maximum number of anti-angiogenic units for the TSPf is about 6, one can increase this concatameric number by deleting the sequences that do not have any anti-angiogenic effects, such as shown below:

15 ATG (CTGAGGCGGCCTCCCCTATGCTATCACAAACGGAGTTCA
GGTAAAAGATCCCCGTGGTCATCTTGTCTGTGACATGTGGTGTGAT
GGTAAAAGAAGTGGTACCCCTGTAGACAAGACAGTGGACACCTCCTCCCCAT) _n T
AA (SEQ ID NO:29),

20 where the corresponding amino acid sequence is:

25 M (LRRPPLCYHNGVQYRNNEEWTDSGKSSPWSSCSVTCGDGVITRIGKSSPWD
ICSVTCGGGV) _n (SEQ ID NO:30),

and wherein n is an integer of from 2 to 24. In a similar manner, the concatameric number of the platelet factor-4 peptide, somatostatin inhibitor, angiostatin, and prolactin can be increased.

30 Since more than one anti-angiogenic pathway exists, concatamers consisting of two or more types of inhibitor are believed to be more effective than the homogenous concatamers. For example, heterogeneous concatamers of TSPI and the fibronectin inhibitors can be inserted into the same vector. An example of a heterogenous concatamer useful is present invention is as follows:

35 ATG (CTGAGGCGGCCTCCCCTATGCTATCACAAACGGAGTTCA
GGTAAAAGATCCCCGTGGTCATCTTGTCTGTGACATGTGGTGTGAT
GGTAAAAGAAGTGGTACCCCTGTAGACAAGACAGTGGACACCTCCTCCCCAT) _x (

40 TATATTGGTTCTCGTGGTAAAAGA) _y TAA (SEQ ID NO:31).

45 The first parenthetical represents the nucleotide sequence of TSPf, whereas the second parenthetical represents the anti-angiogenic fragment isolated from fibronectin, wherein x and y represent the number of repeats of TSPI and fibronectin, respectively. Again, the number of bases delineated by the summation of x + y will generally not exceed 4400 bases.

50 The above heterogeneous concatamers need not be limited to only anti-angiogenic peptides. For example, the protein angiostatin or the large polypeptide fragment of prolactin can be modified with the above-mentioned genes which encode anti-angiogenic peptides. Again, the concatameric number will vary depending on the number of nucleotide bases of the unit angiogenic inhibitor. In this concatamer of large and small anti-angiogenic inhibitors, the ratio of large to small inhibitors is 0.1 to 0.9, preferably 1:1.

A translational start signal Met, has been included in all of the above peptides; and a transcriptional stop codon (TAA) has been included in all of the above DNA sequences.

55 The *Sall* sites indicated in the above-sequences are a useful cloning tool for insertion of the DNA into BAP vector, which is known to useful in expressing proteins efficiently *in vivo* from the β -actin promoter (Ray et al, *Genes Dev.*, 5:2265-2273 (1991)). Other restriction sites can be incorporated into the DNA for cloning into other vectors.

Other useful vectors for gene therapy which can be employed in the present invention include plasmids with a simian viral promoter, e.g., pZeoSV (Invitrogen); or the CMV promoter, e.g., pcDNA3, pRc/CMV or pcDNA1 (Invitrogen). Plasmids with a CMV promoter may contain an intron 5' of the multiple cloning site (Zhu et al, *supra*). Plasmids contain-

ing the BGH terminator instead of the viral SV40 polyA terminator, e.g., pcDNA3, pRc/CMV, pRc/RSV (Norman et al, IBC's 5th Annual Meeting (1995); and Invitrogen vectors), can also be employed in the present invention so as to increase the expression of the tumor suppressor gene and the anti-angiogenic peptide in cells. As stated previously, a vector containing two or more promoters will greatly enhance the therapeutic efficacy. Vectors containing the IRES sequence which allows the translation of two different coding genes to occur from one mRNA transcript will also significantly increase the efficacy of the therapy.

5 Expression of the DNA encoding the tumor suppressor protein and the DNA encoding the anti-angiogenic peptide can be achieved using a variety of promoters, and the particular promoter employed is not critical to the present invention. For example, the promoter can be a generalized promoter, such as the β -actin promoter, a simian viral promoter, 10 or the CMV promoter, or a tissue specific promoter, such as the α -fetal protein promoter which is specific for liver (Kaneko et al, *Cancer Res.*, 55:5283-5287 (1995), the tyrosinase promoter which is specific for melanoma cells (Hughes et al, *Cancer Res.*, 55:3339-3345 (1995); or the enolase promoter which is specific for neurons (Andersen et al, *Cell. Molec. Neurobiol.*, 13:503-515 (1993)).

15 The particular amount of DNA included in the cationic liposomes of the present invention is not critical. Generally, the amount of total DNA is in the range of about 0.005 to 0.32 μ g/pM of liposome, preferably 0.045 to 0.08 μ g/pM of liposome.

20 The DNA encoding a tumor suppressor gene is generally present in an amount of from 0.0025 to 0.16 μ g/pM of liposome, preferably 0.028 to 0.04 μ g/pM of liposome. The DNA encoding an anti-angiogenic peptide is generally present in an amount of from 0.0025 to 0.16 μ g/pM of liposome, preferably 0.028 to 0.04 μ g/pM of liposome.

25 The mole ratio of the DNA encoding the tumor suppressor gene to the DNA encoding the anti-angiogenic peptide is not critical to the present invention. Generally, the mole ratio is between 1:5 to 5:1, preferably about 1 to 1.

The DNA encoding the tumor suppressor gene and the anti-angiogenic peptide may be contained on the same vector, or on separate vectors.

30 Cationic liposomes are prepared similarly to other liposomes. In brief, the cationic lipid with/or without DOPE are dissolved in a solvent, e.g., chloroform. The lipids are then dried in a round bottom flask overnight on a rotary evaporator. The resulting lipids are then hydrated with sterile water over a 1 hr period so form large multilamellar vesicle liposomes. To decrease the size of the liposomes, one may sonicate or pass the liposomes back and forth through a polycarbonate membrane. The DNA is then added to a solution containing the liposomes after their formation.

35 In another embodiment, the above-described objects of the present invention have been met by a method for inhibiting tumor growth in a subject comprising administering to a tumor-bearing subject a cationic liposome:DNA complex comprising DNA encoding a tumor suppressor gene and DNA encoding an anti-angiogenic peptide.

In a preferred embodiment the cationic liposome:DNA complex additionally comprises DNA encoding a tumor suppressor protein.

35 In a further preferred embodiment the cationic polymer:DNA complex additionally comprises DNA encoding a tumor suppressor protein. In a further preferred embodiment the cationic liposomes in the cationic liposome:DNA complex are comprised of one cationic lipid (i.e. 1,2-dioleoyl-sn-glycero-3-ethyl-phosphocholine, 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine, and 2,3-diol-eyloxy)propyl-N,N,N- tri-methyl-ammonium chloride) and may also be comprised of polyethylimine glycol (i.e., a pegylated lipid-1,2-diacyl-sn-glycero-3-phospho-ethanolamine-N-Poly(ethylene glycol) 2000) and a targeted ligand (the amino acid peptide RGD, ferritin, or antibodies targeted toward to HER2).

40 In a further preferred embodiment of the invention said cationic liposomes in said complexes are comprised of one cationic polymer polyethylimine, polylysine, polyhistidine, and Superfect) and may also be comprised of polyethylene glycol and a targeted ligand (the amino acid peptide RGD, ferritin, or antibodies targeted toward to HER2, CEA).

In a further preferred embodiment said tumor suppressor protein in said complexes is selected from the group consisting of the p53, the p21 and the rb.

45 In a further preferred embodiment said tumor suppressor protein in the said complexes is p53.

In a further preferred embodiment of the invention said anti-angiogenic peptide in the said complexes is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, and SEQ ID NO:30.

50 In a further preferred embodiment the said DNA encoding an anti-angiogenic peptide used in the said cationic liposome:DNA complex is present in an amount of from 0.0025 to 0.16 μ g/pM of liposome.

In a further preferred embodiment the said DNA encoding an anti-angiogenic peptide in the said cationic polymer:DNA complex is present in an amount of from 0.016 to 0.33 μ g/ μ g of polymer.

55 In a further preferred embodiment the said DNA encoding a tumor suppressor protein in the said complexes is present in an amount of from 0.0025 to 0.16 μ g/pM.

In a further preferred embodiment of the present invention said DNA encoding a tumor suppressor protein in the said complexes is present in an amount of from 0.016 to 0.33 μ g/pM.

A further embodiment of the present invention is the provision of the use of a cationic polymer:DNA complex com-

prising DNA encoding an anti-angiogenic peptide for the production of a medicament for inhibiting tumor growth in a subject which preferably comprises administering the same to a tumor-bearing subject.

In a further preferred embodiment of the present invention the said complex in the said use additionally comprises DNA encoding a tumor suppressor protein.

5 In a further preferred embodiment of the present invention the said cationic liposome in the said use is (i.e.-1,2-dioleoyl-sn-glycero-3-ethylphosphocholine, 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine, and 2,3-diol-eyloxy)propyl-N,N,N-trimethylammonium chloride and may also be comprised of polyethylene glycol (i.e., a pegylated lipid-1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[Poly-(ethylene glycol) 2000] and a targeted ligand (the amino acid peptide RGD, ferritin, or antibodies targeted toward to HER2).

10 In a further preferred embodiment of the present invention the said cationic polymer in the said use is (i.e. (polyethylenimine Polycat57, polylysine, polyhistidine, and Superfect) and may also be comprised of polyethylene glycol and a targeted ligand (the amino acid peptide RGD, ferritin, or antibodies targeted toward to HER2).

In a further preferred embodiment of the present invention the said tumor suppressor protein used in the said cationic polymer is selected from the group consisting of p53, the p21 and the rb.

15 In a further preferred embodiment of the present invention the said tumor suppressor protein used in the said cationic polymer is p53.

In a further preferred embodiment the said anti-angiogenic peptide used for the said cationic polymer is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ 20 ID NO:27, and SEQ ID NO:30.

In a further preferred embodiment of the present invention the said DNA encoding an anti-angiogenic peptide used in the cationic complex is present in an amount from 0.0025 to 0.16 µg/pM of liposome or 0.016 to 0.33 µg/µg of polymer.

25 In a further preferred embodiment of the present invention the said DNA encoding a tumor suppressor protein used in the said complex is present in an amount of from 0.0025 to 0.16 µg/pM of liposome or 0.016 to 0.33 µg/µg of polymer.

In a further embodiment of the present invention the said cationic polymer:DNA complex comprising a plasmid with one or more promoters expressing either the same gene product (SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, and SEQ ID NO:30) or combinations of these gene products.

30 In a further embodiment of the present invention the cationic liposome:DNA complex comprising a plasmid with an intervening internal ribosomal entry site sequence between two genes (SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, and SEQ ID NO:29) that are either the same or different.

35 In a further preferred embodiment the said anti-angiogenic protein in the said cationic liposome:DNA complex or the said cationic polymer:DNA complex is secretory from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, and SEQ ID NO:30.

The particular type of tumor which can be treated in the present invention is not critical thereto. Examples of tumors which can be treated in accordance with the present invention include solid tumors, e.g., lung, colon, brain, breast and 40 melanoma tumors. All of these tumors are very dependent on blood supply to sustain their growth.

The particular mode of administering the cationic liposome:DNA complex of the present invention is not critical thereto. Examples of such modes include intravenous, subcutaneous and intratumoral injections. Intravenous injection is the preferred administration mode since there is better distribution to the developing blood vessels of the tumor.

45 The amount of cationic liposome:DNA complex to be administered will vary depending upon the age, weight, sex of the subject, as well as the tumor volume and rate of tumor growth in the subject. Generally, the amount to be administered will be about 5 to 60 µg, preferably about 9 to 16 µg.

The following examples are provided for illustrative purposes only, and are in no way intended to limit the scope of the present invention.

50 **EXAMPLE 1**

Production of DNA Vectors

A. TSPI Vector

55 The coding region of the TSPI gene is well-known (GB Accession code-X14787). The TSPI gene was inserted into the *Xba*I site of BAP vector (Ray et al, *supra*), so as to give rise to TSPI vector, wherein expression of the TSPI gene is controlled by the β-actin promoter.

More specifically, TSPI cDNA and Bluescript plasmid (Promega) were digested with *Hind*I and *Xba*I, and then the TSPI cDNA was ligated into Bluescript. Next, Bluescript containing the TSP cDNA and BAP vector were digested with *Sa*I and *Bam*HI, and TSPI cDNA inserted in the *Xba*I site of BAP vector. The correct orientation of the TSPI gene in BAP vector was confirmed by DNA sequencing.

5

B. TSPf Vector

TSPf vector is a vector containing a DNA fragment of the TSPI gene which has the two anti-angiogenic domains (nucleotides 992-1650) (Tolsma et al, *supra*), and a start codon and a stop codon.

10

The DNA fragment was prepared by PCR using thrombospondin I cDNA as template, and 100 pmoles of each of the following primers 5'-TAGGTCTAGAATGACTGAAGAGAACAAAGAG-3' (SEQ ID NO:24); and 5'-ATGGTCTAGATT-AGAGACGACTACGTTCTG-3' (SEQ ID NO:25) so as to amplify nucleotides 1013 to 1650 of the TSPI gene. Both primers contain *Xba*I sites (underlined), the first primer contains an ATG start codon (in bold), and the second primer contains a TTA stop codon (in bold).

15

The resulting 638 base pair fragment of the TSPI gene (hereinafter "TSPf") encodes the peptides that are known to be angiogenic inhibitors (Tolsma et al, *supra*).

After amplification, the DNA fragment was purified, digested with *Xba*I, and the digested fragment inserted into the *Xba*I site of BAP vector such that the expression of the TSPf gene was controlled by the β -actin promoter (Ray et al, *supra*; and Lesoon-Wood et al, *Human Gene Ther.*, 6:395-405 (1995)). The correct orientation of the fragment in BAP vector was verified by digestion with *Bam*HI, and confirmed by DNA sequencing.

20

C. p53 Vector

The coding sequence of the p53 gene was cut from plasmid p1SVhp53c62 (Zakut-Houri et al, *EMBO J.*, 4:1251-1255 (1985)) with *Xba*I, and inserted into the multiple cloning sites of pGEM3Z vector (Promega, Madison, WI). Digestion of the resulting vector with *Sa*I and *Bam*HI generated a 1900 bp fragment that was then inserted into the *Sa*I and *Bam*HI sites of BAP vector such that expression of the p53 gene was controlled by the β -actin promoter. The correct orientation of the p53 gene in BAP vector was confirmed by DNA sequencing.

EXAMPLE 2

Preparation of Cationic Liposome:DNA Complexes

A DOTMA:DOPE liposome mixture is known to efficiently transfect endothelial cells *in vitro* (Tilkins et al, *Focus*, 16:117-119 (1994)). Accordingly, liposome:DNA complexes were prepared using DOTMA:DOPE, in a 1:1 ratio, essentially as described by Debs et al, *J. Biol. Chem.*, 265:10189-10192 (1990). Similar liposomes preparations can be prepared by mixing, at a 1:1 ratio, DOPE with other cationic lipids, such as, 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine, and 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine.

More specifically, a mixture of 400 nmoles of the DOTMA and DOPE were dried overnight on a rotary evaporator. Then, the lipids were rehydrated with 1.5 ml of water for 2 hrs. Next, the milky liposome preparation was sonicated with a bath sonicator until clear. The resulting liposome preparation was then passed through a 50 nm polycarbonate filter between 15 to 20 times with a LipsoFast-Basic extruder (Avestin, Ottawa, On).

The DNA employed was either (1) empty BAP vector; (2) TSPI vector alone; (3) TSPf vector alone; (4) p53 vector alone; (5) p53 vector + TSPI vector; or (6) p53 vector + TSPf vector.

The DNA was prepared with the maxi Qiagen kits (Qiagen Inc., Chatsworth, Ca), and washed twice in 70% (v/v) ethanol. The DNA was then dialyzed against water for 24 hrs to removed any remaining salt.

About 400 pmols of the liposome preparation was gently mixed with between 18 to 35 μ g of total DNA in an Eppendorf tube. This amount in each eppendorf tube was sufficient for two injections. The same amount of DNA was injected in the combination therapies as in the single treatment regimens. For example, if 16 μ g of DNA in the combination therapy (8.0 μ g of p53 + 8.0 μ g of TSPf) was injected into each mouse of one group, then 16 μ g of p53 was injected into each mouse of a second group.

EXAMPLE 3

Anti-Angiogenic Effect of Cationic Liposome:DNA Complexes

The anti-angiogenic effects of the cationic liposome:DNA complexes obtained in Example 2 were evaluated in mice containing MDA-MB-435 breast cancer tumors (American Type Tissue Culture, Bethesda, MD), which are p53 defi-

cient.

More specifically, after administering the anesthetic, Metofane, to 126 female athymic nude mice (NCI), the mice were injected with 2.0×10^5 MDA-MB-435 tumor cells into the mammary fat pad using a stepper (Tridak) and a 27.5 g needle. Two weeks later, the mice whose tumors grew were divided into various treatment regimens, 18 mice per each regimen. The treatment regimens were as follows:

(1) untreated; (2) empty BAP vector; (3) TSPI vector alone; (4) TSPf vector alone; (5) p53 vector alone; (6) p53 vector + TSPI vector; and (7) p53 vector + TSPf vector. The mice received two intravenous injections, the first injection 14 days after the malignant cells had been implanted into the mice, and the second injection 24 days after the malignant cells had been implanted into the mice. The first injection consisted of 200 pmoles of the liposomes complexed with 16 μ g of total DNA. The second injection consisted of 200 pmols of the liposomes complexed with 8.0 μ g of total DNA. The sizes of the tumors were measured 7 days after the second injection. The results are shown in Table 1 below.

TABLE 1

| Anti-tumor Effects of TSPI and TSPf | |
|-------------------------------------|------------------------------|
| Putative Anti-tumor Genes | Tumor Size (mm^3) |
| Untreated | 113.5 \pm 6.41 |
| BAP | 102.9 \pm 6.83 |
| TSPI | 103.2 \pm 8.96 |
| TSPf | 89.4 \pm 11.06 |
| p53 | 80.1 \pm 12.7* |
| p53 + TSPI | 82.9 \pm 6.95* |
| p53 + TSPf | 53.2 \pm 8.37** |

* p53 or p53 + TSPI vs. untreated, $p<0.05$

** p53 + TSPf vs. untreated or BAP, $p<0.01$

As shown in Table 1 above, the p53-treated group was found to be statistically different from the untreated group ($p<0.05$) after 2 injections. However, the p53 treated group was not statistically different from the empty BAP vector group. This was similar to the results described by Lesoon-Wood et al, *Human Gene Ther.*, 6:395-406 (1995), in which p53 was not statistically different from the empty BAP vector group until after 5 injections.

However, p53 in combination with TSPf reduced tumors more effectively than p53 alone. That is, after just 2 injections of this combination therapy, there was a 35% further reduction in tumor growth compared to p53 alone. The combination group was statistically different from both the untreated and the empty BAP vector groups ($p<0.01$). Although TSPf by itself was slightly less effective than p53, TSPf was, unexpectedly, substantially more effective than TSPI. In fact, the full length TSPI-treatment group had no more effect than either the empty vector or the untreated groups. This was unexpected for several reasons: 1) both the full length and the fragment of thrombospondin I contained the anti-angiogenic peptide 2) in a previous ex vivo study (Weinstat-Saslow et al, *supra*), full length thrombospondin I was effective in inhibiting tumor growth, and 3) full length thrombospondin I has a secretory sequence presumably so that the secreted protein can inhibit endothelial proliferation, whereas the thrombospondin I fragment does not contain a secretory sequence.

Regardless of whether there is a secretory sequence, one would predict prior to the present invention that the liposome:antiangiogenic gene would not be an effective antitumor therapy. As taught by Lesoon-Wood et al., the transfection efficiency of the tumor with cationic liposomes was very low. In fact, it could not be quantitated with a primer extension method. We know from the teaching of Weinstat-Saslow et al. that high levels of expressed TSPI in 100% of the tumor cells reduces the tumor growth by only 60% in an ex vivo system. Extrapolating from these findings, a relatively high transfection efficiency of 20% with the liposome: antiangiogenic genes would have resulted in a marginal reduction ($20\% / 100\% \times 60\% \text{ reduction} = 12\%$) of the tumor. This amount of tumor reduction would not have resulted in statistical differences with the liposome:antiangiogenic gene complexes. A transfection efficiency of the tumor above 10% would have easily been measurable with a variety of techniques including the primer extension method (used by Lesoon-Wood et al.). It has been determined that the transfection efficiency of the tumor is less than 5% with these cationic liposomes.

Hence, it was clearly unobvious that DNA encoding an anti-angiogenic peptide alone would be an effective anti-

tumor agent *in vivo*, based upon teachings that an anti-angiogenic peptide is an effective anti-tumor agent (Tolsma et al and Bouck et al), and based upon the teachings that DNA encoding a full-length anti-angiogenic protein is an effective anti-tumor agent *ex vivo* (Weinstat-Saslow et al).

5 A second experiment was carried out to determine whether the combination therapy of p53 and TSPf was effective at lower dosages, and to confirm that the combination of p53 and TSPf reduced the tumor size considerably more than p53 alone.

10 More specifically, 36 mice were injected with 2.0×10^5 MDA-MB-435 tumor cells into the mammary fat pad. Two weeks later, the mice whose tumors grew were divided into various treatment regimens, 18 mice per each regimen. The treatment regimens were as follows: (1) empty BAP vector; (2) p53 vector alone, and (3) p53 vector + TSPf vector. The mice were injected intravenously with 200 pmols of the liposomes complexed with 8.0 μ g of total DNA. Subsequently, the mice were treated in the same manner with 200 pmols of the liposomes complexed with 12 μ g of total DNA for the next 4 injections. Ten days elapsed between each injection. The sizes of the tumors were measured before each injection and 7 days after the last injection. The results are shown in Table 2 below:

15

TABLE 2

| Anti-tumor Effects of p53 and TSPf | |
|------------------------------------|------------------------------|
| Putative Anti-tumor Genes | Tumor Size (mm^3) |
| BAP | 855 \pm 345 |
| p53 | 616 \pm 142 |
| p53 + TSPf | 265 \pm 133* |

20

25 * p53 + TSPf vs. BAP, p<0.02

As shown in Table 2 above, the combination therapy with p53 and TSPf was statistically different from BAP, whereas the p53 alone treatment was not. This experiment confirmed that p53 and TSPf were more effective than p53 alone. Furthermore, a different dosage regimen, without an initial booster dose of 16 μ g of DNA as used in the experiment in Table 1, accentuated the difference between the combination treatment and the p53 alone treatments.

30 In table 1, the TSPf treatment group decreased the tumor more than empty vector or untreated groups. However, it was not statistically significant ($p=.07$). We repeated the experiment after injecting a higher dose of DNA and measured the different treatment groups tumors 10 days after the first treatment.

35

Table 3

| Anti-tumor Effects of TSPf | |
|----------------------------|------------------------------|
| Putative Anti-tumor Genes | Tumor Size (mm^3) |
| Untreated | 80.0 \pm 11.2 |
| BAP | 80.4 \pm 4.5 |
| TSPf | 50.7 \pm 4.8* |

40

45 * TSPf vs. BAP, p<0.025

At the higher dose of 19 μ gs of DNA, the TSPf treatment group was statistically different from either the empty vector or the untreated groups.

50 In another experiment demonstrating the efficacy of antiangiogenic genes, various antiangiogenic genes were examined for their antitumor activity. After administering the anesthetic, Metofane, to 126 female athymic nude mice (NCI), the mice were injected with 3.0×10^5 MDA-MB-435 tumor cells into the mammary fat pad using a stepper (Tridak) and a 27.5 g needle. Two weeks later, the mice whose tumors grew were divided into various treatment regimens, 8 mice per each regimen. The treatment regimens were as follows: (1) BAP vector; (2) TSPf vector alone; (3) laminin peptide vector alone; and (4) angiostatin vector alone. The mice received 4 intravenous injections, the first injection was 10 days after the malignant cells had been implanted into the mice, and the remaining injections were thereafter 10 days apart. The injections consisted of 200 pmoles of the liposomes complexed with 12.5 μ g of total DNA. The results are

shown in Table 4 below.

TABLE 4

| Putative Anti-tumor Genes | Tumor Size(mm^3) |
|---------------------------|-----------------------------|
| BAP | 194.7 \pm 11.9 |
| TSPf | 135.9 \pm 11.9* |
| Laminin peptide | 126.4 \pm 8.4* |
| Angiostatin | 95.2 \pm 6.3** |

* TSPf, Laminin peptide, and Angiostatin vs.

BAP, $p < 0.05$

** Angiostatin vs. BAP, $p < 0.01$

As shown in Table 4 above, the cationic liposomes containing only DNA encoding various anti-angiogenic peptides (TSPf, laminin peptide and angiostatin) significantly inhibited tumor growth.

Next, MCF7 cells (American Type Tissue Culture, Bethesda, MD), which are a breast cancer cell line with two normal p53 alleles, were evaluated as described above except that 4.0×10^6 cells were injected into the mice; and the third injection contained 12 μg of the DNA. Each injection was 10 days apart. Nine mice were injected with each of the following treatments except for regimen (1), in which 8 mice were treated: (1) untreated; (2) BAP; (3) p53; and (4) p53 + TSPf. The sizes of the tumors were measured 7 days after the third injection. The results are shown in Table 5 below.

TABLE 5

| Effect of p53 and TSPf on MCF7s Cells | |
|---------------------------------------|------------------------------|
| Putative Anti-tumor Genes | Tumor Size (mm^3) |
| Untreated | 124.6 \pm 7.3 |
| BAP | 136 \pm 16.8 |
| p53 | 83.1 \pm 13.6* |
| p53 + TSPf | 69.0 \pm 13** |

* p53 vs. untreated or BAP, $p < 0.05$

** p53 + TSPf vs. untreated or BAP, $p < 0.01$

As shown in Table 5 above, the most effective therapy against MCF7s was p53 and TSPf. The significance level for the p53 + TSPf therapy was greater than for p53 alone when they were compared against either the untreated or the BAP groups.

The above experiment verifies that p53 and TSPf decreased the MCF7s tumor more than the p53 treated or the untreated groups. 4×10^6 MCF7 cells were injected bilaterally into the mammary fat pads of the 28 nude mice. After two weeks of growth, these mice were randomly divided into four groups: 1) empty vector, 2) p53, 3) p53 + TSPf, and 4) untreated. The mice received one injection of 200 pmoles of liposomes complexed with 14 μg of DNA, and the tumors from the various treatment groups were measured 10 days after the treatment. The results are shown in Table 6 below.

Table 6

| Putative Anti-tumor Genes | Tumor Size (mm ³) |
|---------------------------|-------------------------------|
| Empty vector- | 54.7±4.0 |
| p53 | 45.5±5.0 |
| p53 + TSPf | 33.9±3.6* |
| Untreated | 61.9±8.3 |

*, p53 + TSPf vs Untreated, p<.025

15 As shown in Table 6, the additional reduction of the tumor by the combined use of p53 and TSPf (also in Tables 1, 2, and 4 above) compared to the use of p53 only, suggest that TSPf and p53 have different mechanisms of action. Although this does not preclude that the target of p53 is the vasculature of the tumor, the mechanism of inhibition of the tumor by p53 is not known at present. However, any mechanism of tumor inhibition by p53 and/or thrombospondin I must account for the low transfection efficiency of the tumor. Again, with a liposome complexed to a chloramphenicol 20 acetyltransferase marker, it has been demonstrated that less than 5% of the tumor derived from MDA-MB-435 cells was transfected with the marker gene.

25 Besides p53 and the antiangiogenic fragment of thrombospondin I, we determined that liposomes complexed to DNA encoding the laminin peptide inhibits tumor growth. More specifically, after administering the anesthetic, Metofane, to 24 female athymic nude mice, the mice were injected with 3.0×10^5 MDA-MB-435 tumor cells into the mammary fat pad using a stepper and a 27.5 g needle. Two weeks later, the mice whose tumors grew were divided into various treatment regimens, 8 mice per each regimen. The treatment regimens were as follows: (1) BAP, (2) laminin, and (3) p53 + laminin. The mice were injected intravenously with 200 pmols of the liposomes complexed with 12.5 mgs of total DNA 30 6.25 mg of each vector when a combination was used. The mice then received 3 injections, each 10 days apart. The tumors were measured at the time of each injection and at the time of the last injection. The results are shown in Table 7 below.

TABLE 7

| Putative Anti-tumor genes | Tumor Size (mm ³) |
|---------------------------|-------------------------------|
| BAP | 345 ± 23.5 |
| Laminin peptide | 280 ± 32.4 |
| Laminin peptide + p53 | 192 ± 10.5* |

*, BAP vs. Laminin peptide + p53, p<0.05

45 As shown in Table 7 above, cationic liposomes containing a combination of DNAs encoding laminin peptide + p53 was unexpectedly more effective in reducing tumor growth than when DNA encoding the anti-angiogenic peptide was used alone. Thus, the addition of a tumor suppressor gene, p53, enhances the anti-tumor effect of the anti-angiogenic peptide.

50 Although intravenous injection are preferred, the method of administration of the liposome:DNA complex is not critical. In figure 1, it was found that intratumoral injections are effective, and it also supports that the therapy is effective against tumors other than breast cancer. In this experiment, 18 mice were injected with 3×10^5 C6 glioma cells (rat brain tumors) subcutaneously. Six days after the injections of these cells, the mice were separated into 3 groups: 1)BAP, 2)FLK-DN (a dominant negative receptor), and 3) angiostatin. After the second intratumoral injection, there was a statistical difference between the angiostatin and the BAP groups. Thus, this therapy is effective when given intratumorally and is effective as expected tumors other than breast tumors.

55 It was also found that this liposome: secretory angiostatin construct was more effective than the non-secreted analog. In brief, we injected 24 nude mice with 3×10^5 MDA-MB-435 cells inserted at the 5 prime end of the their construct. Two weeks later the mice were divided into three groups, they received the following therapies intravenously: 1) liposome:BAP, 2) liposome:secreted angiostatin; and 3) liposome:angiostatin. The concentration of DNA injected into the

mice was 14.5 ugs. The mice received one injection of the liposome:DNA complex and their tumors were measured 10 days after the injection.

5 Table 8

| Efficacy of Secretory Angiostatin | |
|-----------------------------------|-------------------------------|
| Therapeutic Genes | Tumor Size (mm ³) |
| Angiostatin | 28.8±2.2 |
| Angiostatin-Secretory | 18.6±1.8* |
| BAP | 30.5±3.3 |

*p<0.05, BAP vs. Angiostatin-secretory

10 As seen in table 8, the secretory angiostatin treatment group was much more effective than the empty vector control or the angiostatin treatment group in reducing the size of the tumor. From this experiment, it is evident that a secretory sequence inserted into the 5' portion of the antiangiogenic inhibitor will increase its efficacy.

15 In vitro assays indicate that cationic polymers will significantly improve the present therapy. When a carrier such as 20 a cationic lipid was used in this *in vitro* assay, the inhibitory effect (of the genes p53, TSPf, and the combination of p53 and TSPf) was marginal whereas another vector, Superfect (a cationic polymer), was much more effective as a carrier. This is because Superfect was 15 times more effective than the cationic liposomes in transfecting endothelial cells with 25 the CAT marker. The cationic liposomes used in this section was DOSPER (Boehringer), which of the 14 lipids tested gave the best results. Included in this panel of 14 lipids that we tested was lipofectin (BRL) which is a mixture of DOTMA/DOPE that we have used in an *in vivo* study. In brief, we plated 1X10⁶ Huvec cells into each well of a 6 well plate. 25uLs of Superfect complexed with 2 ugs of DNA was added to each plate 24 hours after the initial seeding of the cells. 36 hours after the transfection, the cells were lysed and the amount of CAT protein was assayed.

30 Table 9

| Vectors | Activity(DPMs/protein) |
|-----------------------------|------------------------|
| Cationic liposomes with BAP | 31.1±7.2 |
| Cationic liposomes with CAT | 682±129 |
| Superfect with BAP | 21.4±0.458 |
| Superfect with CAT | 10816±687* |

*p<0.001. Superfect-CAT vs Cationic liposome-CAT

40 This experiment clearly demonstrates that this cationic polymer is a superior in the transfection of endothelial cells, which is a likely target of the therapeutic gene. We have found that Superfect is a better transfection agent than cationic liposomes for many different cell lines. Since Superfect which is a cationic polymers is such an efficient carrier of DNA, this underscores possibility that non-viral carriers as a class of carriers will be effective in decreasing the tumor size. As 45 a result, other non-viral carriers besides liposomes should be included in this patent.

45 Transfection of Huvec Cells with various inhibitors was as follows. 1.0 x 10⁵ Huvec cells (Clonetics), a human endothelial cell line, were plated into each well of a 6-well plate, and placed in a CO₂ incubator at 37°C. Twenty-four hours later, the cells were transfected with 25 mL of Superfect (Qiagen), a cationic polymer, complexed to 2.0 mg of various DNA vectors, i.e., (1) BAP vector; (2) p53 vector; (3) TSPf vector; and (4) p53 vector + TSPf vector. After the cells 50 were exposed for 2 hours to this complex at 37°C, the media was removed, and replaced with fresh EGM media (Clonetics, Inc.), containing 10% (v/v) fetal calf serum, and 1.0% (w/v) glutamine, and the cells placed in a CO₂ incubator at 37°C. Twenty-four hours later, the cell number in each plate was determined by the 3-(4,5-dimethylthiazol-2-yl)-(3-carboxymethoxyphenyl)-2,5-di-phenyltetra-zolium bromide (MTS) assay described by Butke et al, *J. Immunol. Methods*, 157:233-240 (1993).

55 The results are shown in Figure 1 attached hereto dealing with the intratumoral injections of liposome:DNA complexes and its effect on the tumor dimension 6 to 12 days after injection.

As shown in Figure 1, it was found that p53, TSPf, and the combination therapy of p53 and TSPf were effective at inhibiting endothelial cells *in vitro*. The combination of p53 and TSPf was the most effective at inhibiting endothelial

cells. There was a close correlation between the therapeutic genes reducing the tumor size *in vivo* and their effects on endothelial cell number *in vitro*.

Figure 2 shows the effect of different treatment groups on endothelial cells *in vitro*. It was found that the percentage of BAP control is significantly decreased when using BAP-p53 and BAPf-TSPf as treatment groups. A further synergistic decrease is achieved when using BAP-p53/TSPf as the treatment group.

5 While the invention has been described in detail, and with reference to specific embodiments thereof, it will be apparent to one of ordinary skill in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

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SEQUENCE LISTING

5 (1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Archibald James MIXSON
 (B) STREET: 1 Baederwood Ct.
 (C) CITY: Rockville
 (D) STATE: Maryland
 (E) COUNTRY: USA
 (F) POSTAL CODE (ZIP): 21201

10 15 (ii) TITLE OF INVENTION: CATIONIC VEHICLE:DNA COMPLEXES AND THEIR
 USE IN GENE THERAPY

(iii) NUMBER OF SEQUENCES: 33

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

20 25 (v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: EP 97112154.6

(2) INFORMATION FOR SEQ ID NO: 1:

30 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 217 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

40 Met Thr Glu Asn Lys Glu Leu Ala Asn Glu Leu Arg Arg Pro Pro Leu
 1 5 10 15

Cys Tyr His Asn Gly Val Gln Tyr Arg Asn Asn Glu Glu Trp Thr Val
 20 25 30

45 Asp Ser Cys Thr Glu Cys His Cys Gln Asn Ser Val Thr Ile Cys Lys
 35 40 45

50 Lys Val Ser Cys Pro Ile Met Pro Cys Ser Asn Ala Thr Val Pro Asp
 50 55 60

Glu Glu Cys Cys Pro Arg Cys Trp Pro Ser Asp Ser Ala Asp Asp Gly
 65 70 75 80

55

Trp Ser Pro Trp Ser Glu Trp Thr Ser Cys Ser Thr Ser Cys Gly Asn
 85 90 95

5 Gly Ile Gln Gln Arg Gly Arg Ser Cys Asp Ser Leu Asn Asn Arg Cys
 100 105 110

Glu Gly Ser Ser Val Gln Thr Arg Thr Cys His Ile Gln Glu Cys Asp
 115 120 125

10 Lys Arg Phe Lys Gln Asp Gly Gly Trp Ser His Trp Ser Pro Trp Ser
 130 135 140

Ser Cys Val Thr Cys Gly Asp Gly Val Ile Thr Arg Ile Thr Leu Cys
 145 150 155 160

15 Asn Ser Pro Ser Pro Gln Met Asn Gly Lys Pro Cys Glu Gly Glu Ala
 165 170 175

Arg Glu Thr Lys Lys Ala Cys Lys Lys Asp Ala Cys Pro Ile Asn Glu
 180 185 190

Gly Trp Gly Pro Trp Ser Pro Trp Asp Ile Cys Ser Val Thr Cys Gly
 195 200 205

20 Gly-Gly-Val-Gln-Lys-Arg-Ser-Arg-Leu
 25 210 215

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
 30 (A) LENGTH: 656 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATGACTGAAG AGAACAAAGA GTTGGCCAAT GAGCTGAGGC GGCCTCCCCCT ATGCTATCAC 60
 AACGGAGTTTC AGTACAGAAA TAACGAGGAA TGGACTGTTG ATAGCTGCAC TGAGTGTAC 120
 45 TGTCAGAACT CAGTTACCAT CTGCAAAAAG GTGTCCTGCC CCATCATGCC CTGCTCCAAT 180
 GCCACAGTTC CTGATGGAGA ATGCTGTCCCT CGCTGTTGGC CCAGCGACTC TGCGGACGAT 240
 GGCTGGTCTC CATGGTCCGA GTGGACCTCC TGTTCTACGA GCTGTGGCAA TGGAATTCA 300
 50 CAGCGCGGCC GCTCCTGCGA TAGCCTCAAC AACCGATGTG AGGGCTCCTC GGTCCAGACA 360
 CGGACCTGCC ACATTCAAGGA GTGTGACAAA AGATTAAAC AGGATGGTGG CTGGAGCCAC 420

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| | | | | | | |
|------------|-------------|------------|-------------|------------|------------|-----|
| TGGTCCCCGT | GGTCATCTTG | TTCTGTGACA | TGTGGTGATG | GTGTGATCAC | AAGGATCCGG | 480 |
| CTCTGCAACT | CTCCCAGCCC | CCAGATGAAT | GGGAAACCCCT | GTGAAGGCGA | AGCGCGGGAG | 540 |
| ACCAAAGCCT | GCAAGAAAAGA | CGCCTGCCCC | ATCAATGGAG | GCTGGGGTCC | TTGGTCACCA | 600 |
| TGGGACATCT | GTTCTGTCAC | CTGTGGAGGA | GGGGTACAGA | AACGTAGTCT | CTCTAA | 656 |

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 439 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS :

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Met Thr Glu Asn Lys Glu Leu Ala Asn Glu Leu Arg Arg Pro Pro Leu
1 5 10 15

Cys Tyr His Asn Gly Val Gln Tyr Arg Asn Asn Glu Glu Trp Thr Val
 20 25 30

Asp Ser Cys Thr Glu Cys His Cys Gln Asn Ser Val Thr Ile Cys Lys
 35 40 45

Lys Val Ser Cys Pro Ile Met Pro Cys Ser Asn Ala Thr Val Pro Asp
50 55 60

Glu Glu Cys Cys Pro Arg Cys Trp Pro Ser Asp, Ser Ala Asp Asp Gly
 65 70 75 80

Trp Ser Pro Trp Ser Glu Trp Thr Ser Cys Ser Thr Ser Cys Gly Asn
85 90 95

Gly Ile Gln Gln Arg Gly Arg Ser Cys Asp Ser Leu Asn Asn Arg Cys
100 105 110

Lys Arg Phe Lys Gln Asp Gly Gly Trp Ser His Trp Ser Pro Trp Ser
130 135 140

Ser Cys Val Thr Cys Gly Asp Gly Val Ile Thr Arg Ile Thr Leu Cys
145 150 155 160

Asn Ser Pro Ser Pro Gln Met Asn Gly Lys Pro Cys Glu Gly Glu Ala
165 170 175

Gly Trp Gly Pro Trp Ser Pro Trp Asp Ile Cys Ser Val Thr Cys Gly
 195 200 205
 5 Gly Gly Val Gln Lys Arg Ser Arg Leu Cys Val His Ser Arg Met Thr
 210 215 220
 Glu Asn Lys Glu Leu Ala Asn Glu Leu Arg Arg Pro Pro Leu Cys Tyr
 225 230 235 240
 10 His Asn Gly Val Gln Tyr Arg Asn Asn Glu Glu Trp Thr Val Asp Ser
 245 250 255
 Cys Thr Glu Cys His Cys Gln Asn Ser Val Thr Ile Cys Lys Lys Val
 15 260 265 270
 Ser Cys Pro Ile Met Pro Cys Ser Asn Ala Thr Val Pro Asp Glu Glu
 275 280 285
 20 Cys Cys Pro Arg Cys Trp Pro Ser Asp Ser Ala Asp Asp Gly Trp Ser
 290 295 300
 Pro Trp Ser Glu Trp Thr Ser Cys Ser Thr Ser Cys Gly Asn Gly Ile
 305 310 315 320
 25 Gln Gln Arg Gly Arg Ser Cys Asp Ser Leu Asn Asn Arg Cys Glu Gly
 325 330 335
 Ser Ser Val Gln Thr Arg Thr Cys His Ile Gln Glu Cys Asp Lys Arg
 340 345 350
 30 Phe Lys Gln Asp Gly Gly Trp Ser His Trp Ser Pro Trp Ser Ser Cys
 355 360 365
 Val Thr Cys Gly Asp Gly Val Ile Thr Arg Ile Thr Leu Cys Asn Ser
 370 375 380
 35 Pro Ser Pro Gln Met Asn Gly Lys Pro Cys Glu Gly Glu Ala Arg Glu
 385 390 395 400
 Thr Lys Lys Ala Cys Lys Lys Asp Ala Cys Pro Ile Asn Glu Gly Trp
 40 405 410 415
 40 Gly Pro Trp Ser Pro Trp Asp Ile Cys Ser Val Thr Cys Gly Gly Gly
 420 425 430
 Val Gln Lys Arg Ser Arg Leu
 45 435

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1326 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

50

55

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

| | | |
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| | TGTCAGAACT CAGTTACCAT CTGCAAAAG GTGTCCTGCC CCATCATGCC CTGCTCCAAT | 180 |
| 15 | GCCACAGTTC CTGATGGAGA ATGCTGTCCT CGCTGTTGGC CCAGCGACTC TGCGGACGAT | 240 |
| | GGCTGGTCTC CATGGTCCGA GTGGACCTCC TGTCTACGA GCTGTGGCAA TGGAAATTCA | 300 |
| 20 | CAGCCGCGCC GCTCCTGCAG TAGCCTCAAC AACCGATGTG AGGGCTCCTC GGTCCAGACA | 360 |
| | CGGACCTGCC ACATTCAAGGA GTGTGACAAA AGATTTAAC AGGATGGTGG CTGGAGGCCAC | 420 |
| | TGGTCCCCGT GGTCACTCTTG TTCTGTGACA TGTGGTGATG GTGTGATCAC AAGGATCCGG | 480 |
| 25 | CTCTGCAACT CTCCCAGCCC CCAGATGAAT GGGAAACCT GTGAAGGGCGA AGCCGGGAG | 540 |
| | ACCAAAGCCT GCAAGAAAAGA CGCCTGCCCT ATCAATGGAG GCTGGGGTCC TTGGTCACCA | 600 |
| | TGGGACATCT GTTCTGTCAC CTGTGGAGGA GGGGTACAGA AACGTAGTCG TCTCTGCGTC | 660 |
| 30 | GACTCTAGAA TGACTGAAGA GAACAAAGAG TTGGCCAATG AGCTGAGGCG GCCTCCCTA | 720 |
| | TGCTATCACA ACGGAGTTCA GTACAGAAAT AACGAGGAAT GGACTGTTGA TAGCTGCACT | 780 |
| | GAGTGTCACT GTCAGAACTC AGTTACCATC TGCAAAAAGG TGTCCTGCC CATCATGCC | 840 |
| 35 | TGCTCCAATG CCACAGTTCC TGATGGAGAA TGCTGTCCTC GCTGTTGGCC CAGCGACTCT | 900 |
| | GCGGACGATG GCTGGTCTCC ATGGTCCGAG TGGACCTCCT GTTCTACGAG CTGTGGCAAT | 960 |
| 40 | GGAATTCAAGC AGCGCGGCCG CTCCCTGCGAT AGCCTCAACA ACCGATGTGA GGGCTCCTCG | 1020 |
| | GTCCAGACAC GGACCTGCCA CATTCAAGGAG TGTGACAAAA GATTTAAACA GGATGGTGGC | 1080 |
| | TGGAGCCACT GGTCCCCGTG GTCATCTTGT TCTGTGACAT GTGGTGATGG TGTGATCACA | 1140 |
| 45 | AGGATCCGGC TCTGCAACTC TCCCAGCCCC CAGATGAATG GGAAACCTG TGAAGGCGAA | 1200 |
| | GCGCGGGAGA CCAAAGCCTG CAAGAAAGAC GCCTGCCCA TCAATGGAGG CTGGGGTCC | 1260 |
| 50 | TGGTCACCAT GGGACATCTG TTCTGTCACC TGTGGAGGAG GGGTACAGAA ACGTAGTCGT | 1320 |
| | CTCTAA | 1326 |

5 (2) INFORMATION FOR SEQ ID NO: 5:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

20 Met Tyr Ile Gly Ser Arg
1 5

25 (2) INFORMATION FOR SEQ ID NO: 6:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (genomic)

40 (iii) HYPOTHETICAL: NO

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

50 GTCGACATGT ATATTGGTTC TCGTTAAGTC GAC

33

55 (2) INFORMATION FOR SEQ ID NO: 7:

60 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

65 (ii) MOLECULE TYPE: protein

70 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

75 Met Tyr Ile Gly Ser Arg Gly Lys Ser Tyr Ile Gly Ser Arg Gly Lys
1 5 10 15

80 Ser Tyr Ile Gly Ser Arg Gly Lys Ser
20 25

85 (2) INFORMATION FOR SEQ ID NO: 8:

90 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 90 base pairs

55

5 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

| | |
|--|----|
| GTGACATGT ATATTGGTTC TCGTGTAAAA GATATATTGG TTCTCGTGGT AAAAGATATA | 60 |
| TTGGTTCTCG TGGTAAAGA TAAGTCGACC | 90 |

15 (2) INFORMATION FOR SEQ ID NO: 9:

20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 13 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: protein

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

| | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Leu | Tyr | Lys | Lys | Ile | Ile | Lys | Lys | Leu | Leu | Glu | Ser |
| 1 | | | | | 5 | | | | | | | |

30 (2) INFORMATION FOR SEQ ID NO: 10:

35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 54 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

| | |
|--|----|
| GTGACATGC TTTATAAGAA GATCATCAAG AAGCTTCTTG AGAGTTAAGT CGAC | 54 |
|--|----|

50 (2) INFORMATION FOR SEQ ID NO: 11:

55 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 46 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: protein

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

10 Met Leu Tyr Lys Lys Ile Ile Lys Lys Leu Leu Glu Ser Gly Lys Ser
1 5 10 15

10 Leu Tyr Lys Lys Ile Ile Lys Lys Leu Leu Glu Ser Gly Lys Ser Leu
20 25 30

Tyr Lys Lys Ile Ile Lys Lys Leu Leu Glu Ser Gly Lys Ser
35 40 45

15 (2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 153 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: DNA (genomic)

25 (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

30 GTCGACATGC TTTATAAGAA GATCATCAAG AAGCTTCTTG AGAGTGGTAA AAGACTTTAT 60

30 AAGAAGATCA TCAAGAAGCT TCTTGAGAGT GGTAAAAGAC TTTATAAGAA GATCATCAAG 120

AAGCTTCTTG AGAGTGGTAA AAGATAAGTC GAC 153

35 (2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: protein

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Met Phe Cys Tyr Trp Lys Val Cys Trp
1 5

50 (2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid

(C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GTCGACATGT TCTTGTATTG GAAGGGATTG TGGTAAGTCG AC

42

(2) INFORMATION FOR SEQ ID NO: 15:

15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 33 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

25 Met Phe Cys Tyr Trp Lys Val Cys Trp Gly Lys Ser Phe Cys Tyr Trp
 1 5 10 15

Lys Val Cys Trp Gly Lys Ser Phe Cys Trp Lys Val Cys Trp Gly Lys
 20 25 30

30 Ser

(2) INFORMATION FOR SEQ ID NO: 16:

35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 117 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GTCGACATGT TCTTGTATTG GAAGGGATTG TGGGTAAAA GATTCTTGTATTTGAAAGGGAA

60

TTGTGGGGTA AAAGATTCTT GTATTGGAAG GGATTGTGGG GTAAAAGATA AGTCGAC

117

50 (2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

55

5
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

10
(ii) MOLECULE TYPE: protein

15
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Met Gly Arg Gly Asp
1 5

20
(2) INFORMATION FOR SEQ ID NO: 18:

25
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30
(ii) MOLECULE TYPE: DNA (genomic)

35
(iii) HYPOTHETICAL: NO

40
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

45
GTCGACATGT CTTTGTCTTG GAAGACTTTG ACTTAAGTCG AC

42

50
(2) INFORMATION FOR SEQ ID NO: 19:

55
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

60
(ii) MOLECULE TYPE: protein

65
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

70
Met Gly Arg Gly Asp Gly Lys Ser Gly Arg Gly Asp Gly Lys Ser Gly
1 5 10 15

75
Arg Gly Asp Gly Lys Ser
20

80
(2) INFORMATION FOR SEQ ID NO: 20:

85
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 81 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

90
55

(ii) MOLECULE TYPE: DNA (genomic)

5 (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

| | | |
|----|--|----|
| 10 | GTCGACATGG GTCGTGGTGA TGGTAAAAGA GGTCTGGTG ATGGTAAAAG AGGTCGTGGT | 60 |
| | GATGGTAAAA GATAAGTCGA C | 81 |

(2) INFORMATION FOR SEQ ID NO: 21:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 310 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

| | | |
|----|---|--|
| 25 | Met Val Tyr Leu Ser Glu Cys Lys Thr Gly Ile Gly Asn Gly Tyr Arg | |
| | 1 5 10 15 | |

| | | |
|----|---|--|
| 30 | Gly Thr Met Ser Arg Thr Lys Ser Gly Val Ala Cys Gln Lys Trp Gly | |
| | 20 25 30 | |

| | | |
|----|---|--|
| 35 | Ala Thr Phe Pro His Val Pro Asn Tyr Ser Pro Ser Thr His Pro Asn | |
| | 35 40 45 | |

| | | |
|----|---|--|
| 35 | Glu Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Glu Gln | |
| | 50 55 60 | |

| | | |
|----|---|--|
| 40 | Gly Pro Trp Cys Tyr Thr Asp Pro Asp Lys Arg Tyr Asp Tyr Cys | |
| | 65 70 75 80 | |

| | | |
|----|---|--|
| 45 | Asn Ile Pro Glu Cys Glu Glu Cys Met Tyr Cys Ser Gly Glu Lys | |
| | 85 90 95 | |

| | | |
|----|---|--|
| 50 | Tyr Glu Gly Lys Ile Ser Lys Thr Met Ser Gly Lys Asp Cys Gln Ala | |
| | 100 105 110 | |

| | | |
|----|---|--|
| 55 | Trp Asp Ser Gln Ser Pro His Ala His Gly Tyr Ile Pro Ala Lys Phe | |
| | 115 120 125 | |

| | | |
|----|---|--|
| 55 | Pro Ser Lys Asn Leu Lys Met Asn Tyr Cys His Asn Pro Asp Gly Glu | |
| | 130 135 140 | |

| | | |
|----|---|--|
| 55 | Pro Arg Pro Trp Cys Phe Thr Thr Asp Pro Thr Lys Arg Trp Glu Tyr | |
| | 145 150 155 160 | |

Cys Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Pro Pro Ser Pro Thr
 165 170 175
 5 Tyr Gln Cys Leu Lys Gly Arg Gly Glu Asn Tyr Arg Gly Thr Val Ser
 180 185 190
 Val Thr Val Ser Gly Lys Thr Cys Gln Arg Trp Ser Glu Gln Thr Pro
 195 200 205
 10 His Arg His Asn Arg Thr Pro Glu Asn Phe Pro Cys Arg Asn Leu Glu
 210 215 220
 Glu Asn Tyr Cys Arg Asn Pro Asp Gly Glu Thr Ala Pro Trp Cys Tyr
 225 230 235 240
 15 Thr Thr Asp Ser Gln Leu Arg Trp Glu Tyr Cys Glu Ile Pro Ser Cys
 245 250 255
 Glu Ser Ser Ala Ser Pro Asp Gln Ser Asp Ser Ser Val Pro Pro Glu
 260 265 270
 20 Glu Gln Thr Pro Val Val Gln Glu Cys Tyr Gln Ser Asp Gly Gln Ser
 275 280 285
 Tyr Arg Gly Thr Ser Ser Thr Thr Ile Thr Gly Lys Lys Cys Gln Ser
 290 295 300
 25 Glu Gln Thr Pro His Arg
 305 310

(2) INFORMATION FOR SEQ ID NO: 22:

30 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 645 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 35
 (ii) MOLECULE TYPE: DNA (genomic)
 (iii) HYPOTHETICAL: NO
 40
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:
 GTGACATGG TGTATCTGTC AGAATGTAAG ACCGGCATCG GCAACGGCTA CAGAGGAACC 60
 45 ATGTCCAGGA CAAAGAGTGG TGGTGCCTGT CAAAAGTGGG GTGCCACGTT CCCCCACGTA 120
 CCCAACTACT CTCCCAGTAC ACATCCCAAT GAGGGACTAG AAGAGAACTA CTGTAGGAAC 180
 CCAGACAATG ATGAACAAGG GCCTTGGTGC TACACTACAG ATCCGGACAA GAGATATGAC 240
 50 TACTGCAACA TTCCCTGAATG TGAAGAGGAA TGCATGTACT GCAGTGGAGA AAAGTATGAG 300
 GGCAAAATCT CCAAGACCAT GTCTGGACTT GACTGCCAGG CCTGGGATTG TCAGAGCCCA 360

55

| | | |
|----|---|-----|
| 5 | CATGCTCATG GATACATCCC TGCCAAATT CCAAGCAAGA ACCTGAAGAT GAATTATTGC | 420 |
| | CACAACCCCTG ACGGGGAGCC AAGGCCCTGG TGCTTCACAA CAGACCCAC CAAACGCTGG | 480 |
| | GAATACTGTG ACATCCCCCG CTGCACAACA CCCCCGCCCG CACCCAGCCC AACCTACCAA | 540 |
| 10 | TGTCTGAAAG GAAGAGGTGA AAATTACCGA GGGACCGTGT CTGTCACCGT GTCTGGAAA | 600 |
| | ACCTGTCAGC GCTGGAGTGA GCAAACCCCT CATAGGTGAG TCGAC | 645 |

(2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 623 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

| | |
|---|--|
| Met Val Tyr Leu Ser Glu Cys Lys Thr Gly Ile Gly Asn Gly Tyr Arg | |
| 1 5 10 15 | |

| | |
|---|--|
| Gly Thr Met Ser Arg Thr Lys Ser Gly Val Ala Cys Gln Lys Trp Gly | |
| 20 25 30 | |

| | |
|---|--|
| Ala Thr Phe Pro His Val Pro Asn Tyr Ser Pro Ser Thr His Pro Asn | |
| 35 40 45 | |

| | |
|---|--|
| Glu Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Glu Gln | |
| 50 55 60 | |

| | |
|---|--|
| Gly Pro Trp Cys Tyr Thr Asp Pro Asp Lys Arg Tyr Asp Tyr Cys | |
| 65 70 75 80 | |

| | |
|---|--|
| Asn Ile Pro Glu Cys Glu Glu Cys Met Tyr Cys Ser Gly Glu Lys | |
| 85 90 95 | |

| | |
|---|--|
| Tyr Glu Gly Ile Ser Lys Thr Met Ser Gly Lys Asp Cys Gln Ala | |
| 100 105 110 | |

| | |
|---|--|
| Trp Asp Ser Gln Ser Pro His Ala His Gly Tyr Ile Pro Ala Lys Phe | |
| 115 120 125 | |

| | |
|---|--|
| Pro Ser Lys Asn Leu Lys Met Asn Tyr Cys His Asn Pro Asp Gly Glu | |
| 130 135 140 | |

| | |
|---|--|
| Pro Arg Pro Trp Cys Phe Thr Thr Asp Pro Thr Lys Arg Trp Glu Tyr | |
| 145 150 155 160 | |

| | |
|---|--|
| Cys Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Pro Pro Ser Pro Thr | |
| 165 170 175 | |

Tyr Gln Cys Leu Lys Gly Arg Gly Glu Asn Tyr Arg Gly Thr Val Ser
 180 185 190
 5 Val Thr Val Ser Gly Lys Thr Cys Gln Arg Trp Ser Glu Gln Thr Pro
 195 200 205
 His Arg His Asn Arg Thr Pro Glu Asn Phe Pro Cys Arg Asn Leu Glu
 210 215 220
 10 Glu Asn Tyr Cys Arg Asn Pro Asp Gly Glu Thr Ala Pro Trp Cys Tyr
 225 230 235 240
 Thr Thr Asp Ser Gln Leu Arg Trp Glu Tyr Cys Glu Ile Pro Ser Cys
 15 245 250 255
 Glu Ser Ser Ala Ser Pro Asp Gln Ser Asp Ser Ser Val Pro Pro Glu
 260 265 270
 20 Glu Gln Thr Pro Val Val Gln Glu Cys Tyr Gln Ser Asp Gly Gln Ser
 275 280 285
 Tyr Arg Gly Thr Ser Ser Thr Thr Ile Thr Gly Lys Lys Cys Gln Ser
 290 295 300
 25 Glu Gln Thr Pro His Arg Gly Lys Ser Met Val Tyr Leu Ser Glu Cys
 305 310 315 320
 Lys Thr Gly Ile Gly Asn Gly Tyr Arg Gly Thr Met Ser Arg Thr Lys
 325 330 335
 30 Ser Gly Val Ala Cys Gln Lys Trp Gly Ala Thr Phe Pro His Val Pro
 340 345 350
 Asn Tyr Ser Pro Ser Thr His Pro Asn Glu Gly Leu Glu Glu Asn Tyr
 355 360 365
 35 Cys Arg Asn Pro Asp Asn Asp Glu Gln Gly Pro Trp Cys Tyr Thr Thr
 370 375 380
 Asp Pro Asp Lys Arg Tyr Asp Tyr Cys Asn Ile Pro Glu Cys Glu Glu
 40 385 390 395 400
 Glu Cys Met Tyr Cys Ser Gly Glu Lys Tyr Glu Gly Lys Ile Ser Lys
 405 410 415
 Thr Met Ser Gly Lys Asp Cys Gln Ala Trp Asp Ser Gln Ser Pro His
 45 420 425 430
 Ala His Gly Tyr Ile Pro Ala Lys Phe Pro Ser Lys Asn Leu Lys Met
 435 440 445
 Asn Tyr Cys His Asn Pro Asp Gly Glu Pro Arg Pro Trp Cys Phe Thr
 50 450 455 460
 Thr Asp Pro Thr Lys Arg Trp Glu Tyr Cys Asp Ile Pro Arg Cys Thr

| | | | | |
|----|---|-----|-----|-----|
| | 465 | 470 | 475 | 480 |
| 5 | Thr Pro Pro Pro Pro Pro Ser Pro Thr Tyr Gln Cys Leu Lys Gly Arg | | | |
| | 485 | 490 | 495 | |
| | Gly Glu Asn Tyr Arg Gly Thr Val Ser Val Thr Val Ser Gly Lys Thr | | | |
| | 500 | 505 | 510 | |
| 10 | Cys Gln Arg Trp Ser Glu Gln Thr Pro His Arg His Asn Arg Thr Pro | | | |
| | 515 | 520 | 525 | |
| | Glu Asn Phe Pro Cys Arg Asn Leu Glu Glu Asn Tyr Cys Arg Asn Pro | | | |
| | 530 | 535 | 540 | |
| 15 | Asp Gly Glu Thr Ala Pro Trp Cys Tyr Thr Asp Ser Gln Leu Arg | | | |
| | 545 | 550 | 555 | 560 |
| | Trp Glu Tyr Cys Glu Ile Pro Ser Cys Glu Ser Ser Ala Ser Pro Asp | | | |
| | 565 | 570 | 575 | |
| 20 | Gln Ser Asp Ser Ser Val Pro Pro Glu Glu Gln Thr Pro Val Val Gln | | | |
| | 580 | 585 | 590 | |
| | Glu Cys Tyr Gln Ser Asp Gly Gln Ser Tyr Arg Gly Thr Ser Ser Thr | | | |
| 25 | | 595 | 600 | 605 |
| | Thr Ile Thr Gly Lys Lys Cys Gln Ser Glu Gln Thr Pro His Arg | | | |
| | 610 | 615 | 620 | |
| | (2) INFORMATION FOR SEQ ID NO: 24: | | | |
| 30 | (i) SEQUENCE CHARACTERISTICS: | | | |
| | (A) LENGTH: 1284 base pairs | | | |
| | (B) TYPE: nucleic acid | | | |
| | (C) STRANDEDNESS: single | | | |
| | (D) TOPOLOGY: linear | | | |
| 35 | (ii) MOLECULE TYPE: DNA (genomic) | | | |
| | (iii) HYPOTHETICAL: NO | | | |
| 40 | (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24: | | | |
| | GTCGACATGG TGTATCTGTC AGAATGTAAG ACCGGCATCG GCAACGGCTA CAGAGGAACC 60 | | | |
| 45 | ATGTCCAGGA CAAAGAGTGG TGTTGCCTGT CAAAAGTGGG GTGCCACGTT CCCCCACGTA 120 | | | |
| | CCCAACTACT CTCCCAGTAC ACATCCAAT GAGGGACTAG AAGAGAACTA CTGTAGGAAC 180 | | | |
| | CCAGACAATG ATGAACAAGG GCCTTGGTGC TACACTACAG ATCCGGACAA GAGATATGAC 240 | | | |
| 50 | TACTGCAACA TTCCTGAATG TGAAGAGGAA TGCATGTACT GCAGTGGAGA AAAGTATGAG 300 | | | |
| | GGCAAAATCT CCAAGACCAT GTCTGGACTT GACTGCCAGG CCTGGGATTG TCAGAGCCCA 360 | | | |

55

| | | |
|----|---|------|
| 5 | CATGCTCATG GATACATCCC TGCCAAATT CCAAGCAAGA ACCTGAAGAT GAATTATTGC | 420 |
| | CACAACCTG ACGGGGAGCC AAGGCCCTGG TGCTTCACAA CAGACCCCAC CAAACGCTGG | 480 |
| | GAATACTGTG ACATCCCCCG CTGCACAACA CCCCCGCC CACCCAGCCC AACCTACCAA | 540 |
| 10 | TGTCTGAAAG GAAGAGGTGA AAATTACCGA GGGACCGTGT CTGTCACCGT GTCTGGAAA | 600 |
| | ACCTGTCAGC GCTGGAGTGA GCAAACCCCT CATAGGGTA AAAGAATGGT GTATCTGTCA | 660 |
| | GAATGTAAGA CCGGCATCGG CAACGGCTAC AGAGGAACCA TGTCCAGGAC AAAGAGTGGT | 720 |
| | GTTGCCTGTC AAAAGTGGGG TGCCACGTT CCCCACGTAC CCAACTACTC TCCCAGTACA | 780 |
| 15 | CATCCAATG AGGGACTAGA AGAGAACTAC TGTAGGAACC CAGACAATGA TGAACAAGGG | 840 |
| | CCTTGGTGCT ACACTACAGA TCCGGACAAG AGATATGACT ACTGCAACAT TCCTGAATGT | 900 |
| | GAAGAGGAAT GCATGTACTG CAGTGGAGAA AAGTATGAGG GCAAATCTC CAAGACCATG | 960 |
| 20 | TCTGGACTTG ACTGCCAGGC CTGGGATTCT CAGAGCCCAC ATGTCATGG ATACATCCCT | 1020 |
| | GCAAATTTC CAAGCAAGAA CCTGAAGATG AATTATTGCC ACAACCCCTGA CGGGGAGCCA | 1080 |
| 25 | AGGCCCTGGT GCTTCACAAC AGACCCCACC AAACGCTGGG AATACTGTGA CATCCCCCGC | 1140 |
| | TGCACAACAC CCCCGCC CACCCAGCCCA ACCTACCAAT GTCTGAAAGG AAGAGGTGAA | 1200 |
| | AATTACCGAG GGACCGTGTC TGTCACCGTG TCTGGAAAA CCTGTCAGCG CTGGAGTGAG | 1260 |
| 30 | CAAACCCCTC ATAGGTGAGT CGAC | 1284 |

(2) INFORMATION FOR SEQ ID NO: 25:

| | | |
|----|---|--|
| 35 | (i) SEQUENCE CHARACTERISTICS: | |
| | (A) LENGTH: 125 amino acids | |
| | (B) TYPE: amino acid | |
| | (C) STRANDEDNESS: | |
| | (D) TOPOLOGY: linear | |
| 40 | (ii) MOLECULE TYPE: protein | |
| | (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25: | |
| 45 | Met Leu Pro Ile Cys Pro Gly Gly Ala Ala Arg Cys Gln Val Thr Leu | |
| | 1 5 10 15 | |
| | Arg Glu Leu Phe Asp Arg Ala Val Val Leu Ser His Tyr Ile His Asn | |
| | 20 25 30 | |
| 50 | Leu Ser Ser Glu Met Phe Ser Glu Phe Glu Lys Arg Tyr Thr His Gly | |
| | 35 40 45 | |
| | Arg Gly Phe Ile Thr Lys Ala Ile Asn Ser Cys His Thr Ser Ser Leu | |
| | 50 55 60 | |

55

Ala Thr Pro Glu Asp Lys Glu Gln Ala Gln Gln Met Asn Gln Lys Asp
 65 70 75 80

5 Phe Leu Ser Leu Ile Val Ser Ile Leu Arg Ser Trp Asn Glu Pro Leu
 85 90 95

Tyr His Leu Val Thr Glu Val Arg Gly Met Gln Glu Ala Pro Gln Ala
 100 105 110

10 Ile Leu Ser Lys Ala Val Glu Ile Glu Glu Gln Thr Lys
 115 120 125

(2) INFORMATION FOR SEQ ID NO: 26:

15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 387 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GTCGACATGT TGCCCATCTG TCCCGCGGG GCTGCCGAT GCCAGGTGAC CCTTCGAGAC 60

30 CTGTTGACC GCGCCGTCGT CCTGTCAC TACATCCATA ACCTCTCCTC AGAAATGTTC 120

AGCGAATTCTG ATAAACGGTA TACCCATGGC CGGGGGTTCA TTACCAAGGC CATCAACAGC 180

TGCCACACTT CTTCCCTTGC CACCCCGAA GACAAGGAGC AAGCCAACA GATGAATCAA 240

35 AAAGACTTTC TGAGCCTGAT AGTCAGCATA TTGCGATCCT GGAATGAGCC TCTGTATCAT 300

CTGGTCACGG AACTACGTGG TATGCAAGAA GCCCCGGAGG CTATCCTATC CAAAGCTGTA 360

40 GAGATTGAGG AGCAAAACCTA AGTCGAC 387

(2) INFORMATION FOR SEQ ID NO: 27:

45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 255 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Met Leu Pro Ile Cys Pro Gly Gly Ala Ala Arg Cys Gln Val Thr Leu
 1 5 10 15

5 Arg Glu Leu Phe Asp Arg Ala Val Val Leu Ser His Tyr Ile His Asn
 20 25 30

Leu Ser Ser Glu Met Phe Ser Glu Phe Glu Lys Arg Tyr Thr His Gly
 35 40 45

10 Arg Gly Phe Ile Thr Lys Ala Ile Asn Ser Cys His Thr Ser Ser Leu
 50 55 60

Ala Thr Pro Glu Asp Lys Glu Gln Ala Gln Gln Met Asn Gln Lys Asp
 65 70 75 80

15 Phe Leu Ser Leu Ile Val Ser Ile Leu Arg Ser Trp Asn Glu Pro Leu
 85 90 95

Tyr His Leu Val Thr Glu Val Arg Gly Met Gln Glu Ala Pro Gln Ala
 20 100 105 110

Ile Leu Ser Lys Ala Val Glu Ile Glu Glu Gln Thr Lys Gly Lys Ser
 115 120 125

25 Met Leu Pro Ile Cys Pro Gly Gly Ala Ala Arg Cys Gln Val Thr Leu
 130 135 140

Arg Glu Leu Phe Asp Arg Ala Val Val Leu Ser His Tyr Ile His Asn
 145 150 155 160

30 Leu Ser Ser Glu Met Phe Ser Glu Phe Glu Lys Arg Tyr Thr His Gly
 165 170 175

Arg Gly Phe Ile Thr Lys Ala Ile Asn Ser Cys His Thr Ser Ser Leu
 180 185 190

35 Ala Thr Pro Glu Asp Lys Glu Gln Ala Gln Gln Met Asn Gln Lys Asp
 195 200 205

Phe Leu Phe Leu Ser Leu Ile Val Ser Ile Leu Arg Ser Trp Asn Glu
 40 210 215 220

Pro Leu Tyr His Leu Val Thr Glu Val Arg Gly Met Gln Glu Ala Pro
 225 230 235 240

45 Gln Ala Ile Leu Ser Lys Ala Val Glu Ile Glu Glu Gln Thr Lys
 245 250 255

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 756 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

| | | |
|----|--|-----|
| 10 | ATGTTGCCCA TCTGTCCCCG CGGGGCTGCC CGATGCCAGG TGACCCCTCG AGACCTGTTT | 60 |
| | GACCGCGCCG TCGTCCTGTC CCACTACATC CATAACCTCT CCTCAGAAAT GTTCAGCGAA | 120 |
| | TTCGATAAAC GGTATAACCA TGGCCGGGGG TTCATTACCA AGGCCATCAA CAGCTGCCAC | 180 |
| 15 | ACTTCTTCCC TTGCCACCCC CGAAGACAAG GAGCAAGCCC AACAGATGAA TCAAAAAGAC | 240 |
| | TTTCTGAGCC TGATAGTCAG CATATTGCGA TCCTGGAATG AGCCTCTGTA TCATCTGGTC | 300 |
| | ACGGAAGTAC GTGGTATGCA AGAAGCCCCG GAGGCTATCC TATCCAAGC TGTAGAGATT | 360 |
| 20 | GAGGAGCAA CCGGTAAAAG AATGTTGCCC ATCTGTCCCCG GCGGGGCTGC CCGATGCCAG | 420 |
| | GTGACCCCTTC GAGACCTGTT TGACCGCGCC GTCGTCCTGT CCCACTACAT CCATAACCTC | 480 |
| | TCCTCAGAAA TGTCAGCGA ATTGATAAA CGGTATAACCC ATGGCCGGGG GTTCATTACC | 540 |
| 25 | AAGGCCATCA ACAGCTGCCA CACTTCTTCC CTTGCCACCC CCGAAGACAA GGAGCAAGCC | 600 |
| | CAACAGATGA ATCAAAAAGA CTTTCTGAGC CTGATAGTCA GCATATTGCG ATCCTGGAAT | 660 |
| 30 | GAGCCTCTGT ATCATCTGGT CACGGAAGTA CGTGGTATGC AAGAAGCCCC GGAGGCTATC | 720 |
| | CTATCCAAAG CTGTAGAGAT TGAGGAGCAA ACCTAA | 756 |

(2) INFORMATION FOR SEQ ID NO: 29:

35

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 161 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

| | | |
|----|--|-----|
| 50 | ATGCTGAGGC GGCCTCCCCCT ATGCTATCAC AACGGAGTTC AGTACAGAAA TAACGGTAAA | 60 |
| | AGATCCCCGT GGTCATTTG TTCTGTGACA TGTGGTGATG GTGTGATGGT AAAAGAAGTG | 120 |
| | GTACCCCTGTA GACAAGACAG TGGACACCTC CTCCCCATTA A | 161 |

55

(2) INFORMATION FOR SEQ ID NO: 30:

5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 63 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

15 Met Leu Arg Arg Pro Pro Leu Cys Tyr His Asn Gly Val Gln Tyr Arg
 1 5 10 15

Asn Asn Glu Glu Trp Thr Val Asp Ser Gly Lys Ser Ser Pro Trp Ser
 20 25 30

20 Ser Cys Ser Val Thr Cys Gly Asp Gly Val Ile Thr Arg Ile Gly Lys
 35 40 45

Ser Ser Pro Trp Asp Ile Cys Ser Val Thr Cys Gly Gly Val
 50 55 60

25 (2) INFORMATION FOR SEQ ID NO: 31:

30 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 185 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (genomic)

35 (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

40 ATGCTGAGGC GGCCTCCCCT ATGCTATCAC AACGGAGTTC AGTACAGAAA TAACGGTAAA 60
 AGATCCCCGT GGTCACTTTG TTCTGTGACA TGTGGTGATG GTGTGATGGT AAAAGAAGTG 120
 GTACCCCTGTA GACAAGACAG TGGACACCTC CTCCCCATTA TATTGGTTCT CGTGGTAAAA 180
 45 GATAA 185

(2) INFORMATION FOR SEQ ID NO: 32:

50 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 31 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

TAGGTCTAGA ATGACTGAAG AGAACAAAGA G

(2) INFORMATION FOR SEQ ID NO: 33:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

ATGGTCTAGA TTAGAGACGA CTACGGTTCT G

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Claims

35 1. A cationic liposome:DNA complex comprising DNA encoding an anti-angiogenic peptide.

2. A cationic polymer:DNA complex comprising the DNA encoding an antiangiogenic peptide.

3. The complex of Claim 1, wherein said complex additionally comprises DNA encoding a tumor suppressor protein.

40 4. The complex of Claim 2, wherein said complex additionally comprises DNA encoding a tumor suppressor protein.

5. The complex of Claim 1, wherein said cationic liposomes are comprised of one cationic lipid (i.e. 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine, 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine, and 2,3-diol-eyloxy)propyl-N,N,N-trimethyl-ammonium chloride) and may also be comprised of polyethylene glycol (i.e., a pegylated lipid-1,2-diacyl-sn-glycero-3-phospho-ethanolamine-N-Poly(ethylene glycol) 2000) and a targeted ligand (the amino acid peptide RGD, ferritin, or antibodies targeted toward to HER2).

45 6. The complex of Claim 1, wherein said cationic liposomes are comprised of one cationic polymer polyethylenimine Polycat57, polylysine, polyhistidine, and Superfect) and may also be comprised of polyethylene glycol and a targeted ligand (the amino acid peptide RGD, ferritin, or antibodies targeted toward to HER2, CEA).

50 7. The complex of Claims 4 or 5, wherein said tumor suppressor protein is selected from the group consisting of the p53, the p21 and the rb.

55 8. The complex of Claims 3 or 4, wherein said tumor suppressor protein is p53.

9. The complex of Claims 1, 2, or 3, wherein said anti-angiogenic peptide is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID

NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, and SEQ ID NO:30.

10. The complex of Claims 1 or 3, wherein said DNA encoding an anti-angiogenic peptide is present in an amount of 5 from 0.0025 to 0.16 µg/pM of liposome.
11. The complex of Claims 2 or 4, wherein said DNA encoding an anti-angiogenic peptide is present in an amount of 10 from 0.016 to 0.33 µg/µg of polymer.
12. The complex of Claim 8, wherein said DNA encoding a tumor suppressor protein is present in an amount of from 15 0.0025 to 0.16 µg/pM.
13. The complex of Claim 8, wherein said DNA encoding a tumor suppressor protein is present in an amount of from 20 0.016 to 0.33 µg/pM.
14. Use of a cationic polymer:DNA complex comprising DNA encoding an anti-angiogenic peptide for the production of 25 a medicament for inhibiting tumor growth in a subject which preferably comprises administering the same to a tumor-bearing subject.
15. The use of Claim 14, where said complex additionally comprises DNA encoding a tumor suppressor protein.
16. The use of claim 1, wherein said cationic liposome (i.e.-1,2-dioleoyl-sn-glycero-3-ethylphosphocholine,1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine, and 2,3-diol-eyloxy)propyl-N,N,N- trimethylammonium chloride and may 30 also be comprised of polyethylene glycol (i.e., a pegylated-lipid-1,2 -diacy-sn-glycero-3-phospho-ethanolamine-N-[Poly-(ethylene glycol) 2000] and a targeted ligand (the amino acid peptide RGD, ferritin, or antibodies targeted toward to HER2).
17. The use of claim 2, wherein said cationic polymer (i.e. (polyethylenimine Polycat57, polylysine, polyhistidine, and Superfect) and may also be comprised of polyethylene glycol and a targeted ligand (the amino acid peptide RGD, ferritin, or antibodies targeted toward to HER2).
18. The use of Claim 15, wherein said tumor suppressor protein is selected from the group consisting of p53, the p21 and the rb.
19. The use of Claim 18, wherein said tumor suppressor protein is p53.
20. The use of Claim 14, wherein said anti-angiogenic peptide is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, and SEQ ID NO:30.
21. The use of Claim 14 or Claim 15, wherein said DNA encoding an anti-angiogenic peptide is present in an amount 40 from 0.0025 to 0.16 µg/pM of liposome or 0.016 to 0.33 µg/µg of polymer.
22. The use of Claims 14 or 15, wherein said DNA encoding a tumor suppressor protein is present in an amount of from 45 0.0025 to 0.16 µg/pM of liposome or 0.016 to 0.33 µg/µg of polymer.
23. A cationic polymer:DNA complex comprising a plasmid with one or more promoters expressing either the same gene product (SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, and SEQ ID NO:30) or combinations of these gene products.
24. A cationic liposome:DNA complex comprising a plasmid with an intervening internal ribosomal entry site sequence 50 between two genes (SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, and SEQ ID NO:29) that are either the same or different.
25. The complex of Claim 1 or Claim 2, wherein said anti-angiogenic protein is secretory from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID

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NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, and SEQ ID NO:30.

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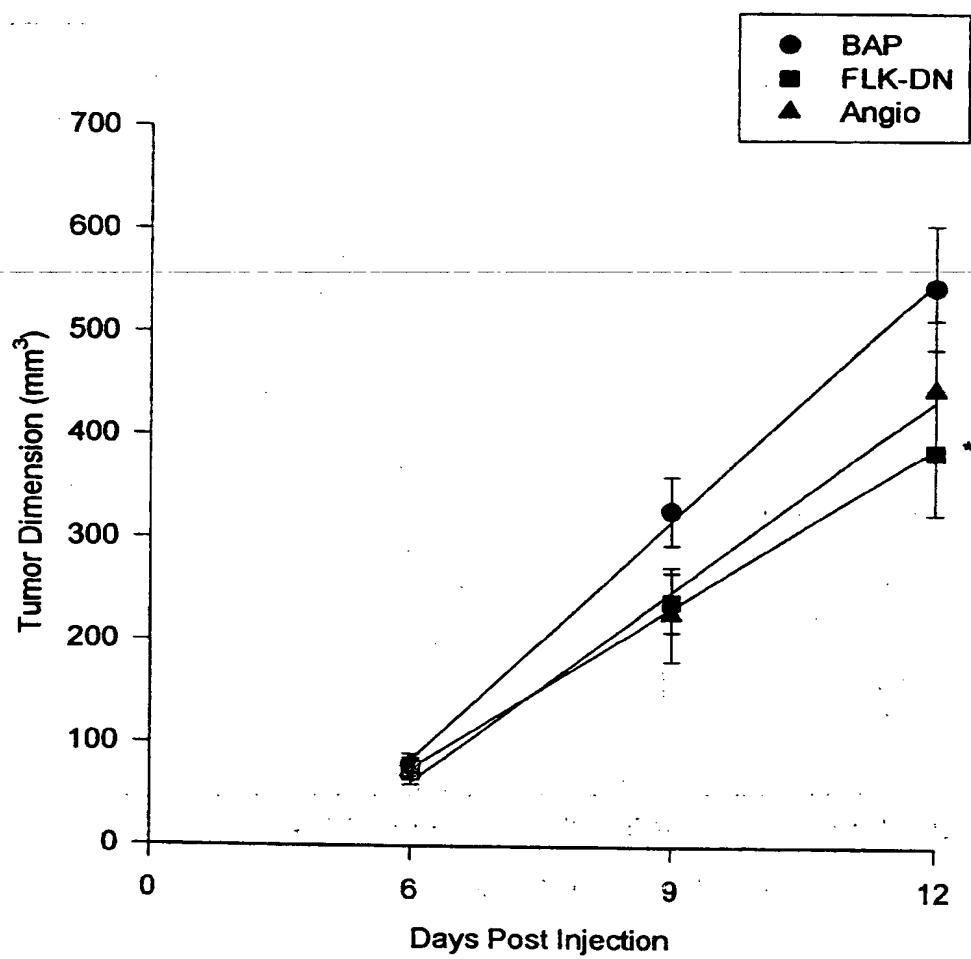
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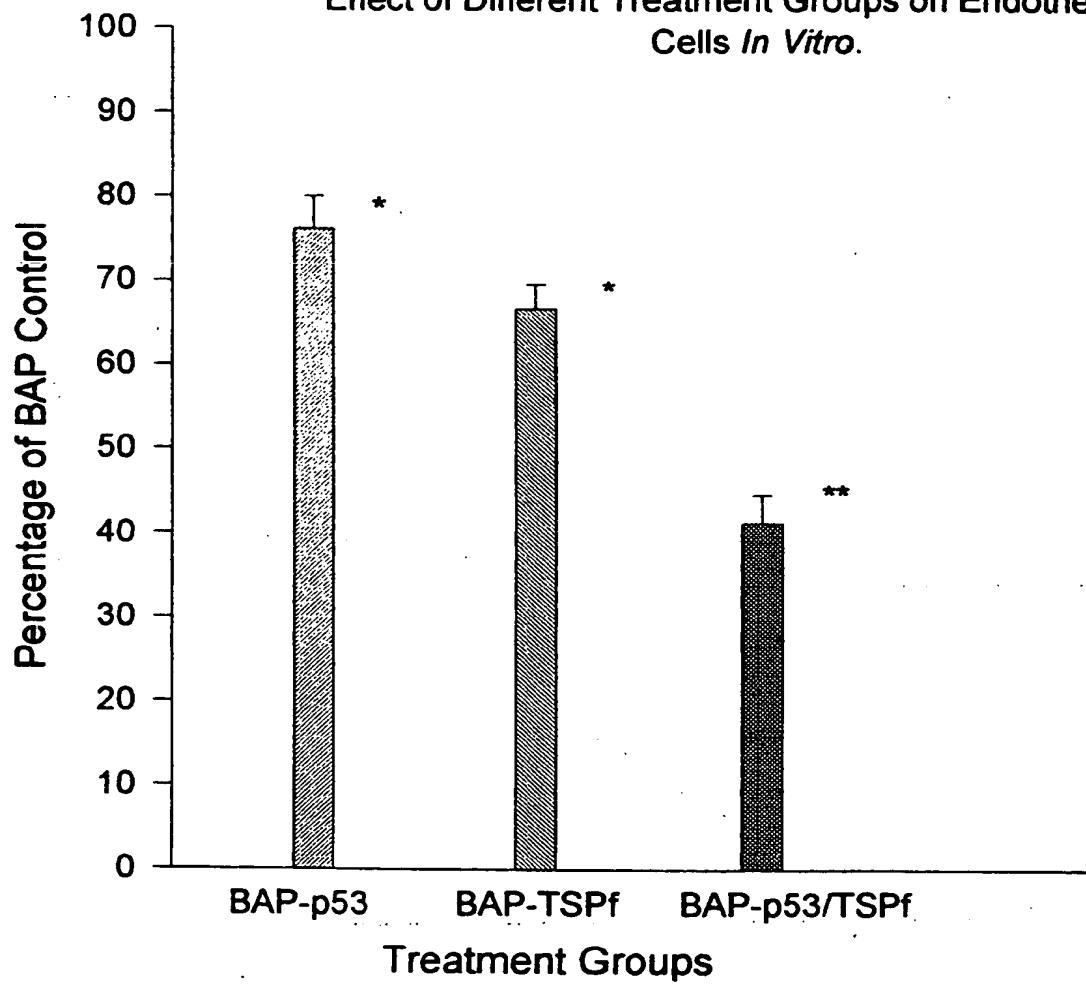
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Figure 1
Intratumoral Injections of Liposome:DNA Complexes



*, Angio vs. BAP, $p < 0.05$

Figure 2
Effect of Different Treatment Groups on Endothelial Cells *In Vitro*.



* - BAP vs BAP-p53 or BAP-TSPf, $p < 0.05$

** - BAP-p53 or BAP-TSPf vs BAP-p53/BAP-TSPf, $p < 0.01$

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(19)



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(71) Applicant:
Mixson, Archibald James
Rockville, MD 21201 (US)

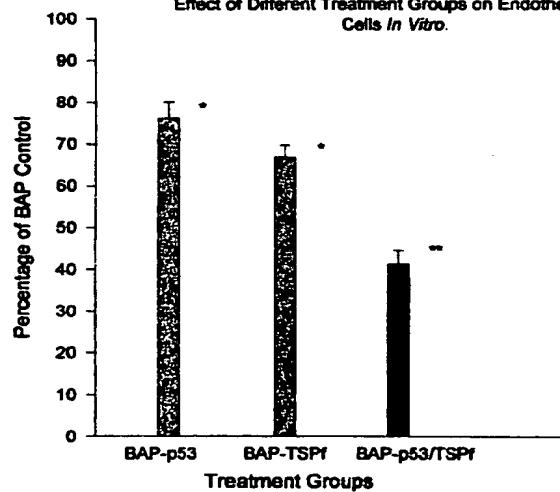
(72) Inventor:
Mixson, Archibald James
Rockville, MD 21201 (US)

(74) Representative:
Sieckmann, Ralf, Dr. et al
Cohausz Hase Dawidowicz & Partner,
Patent- und Rechtsanwaltskanzlei,
Schumannstrasse 97
40237 Düsseldorf (DE)

(54) Cationic vehicle: DNA complexes and their use in gene therapy

(57) Cationic vehicles:DNA complexes comprising DNA encoding an anti-angiogenic peptide or DNA encoding a tumor suppressor protein and DNA encoding an anti-angiogenic peptide, as well as their use in gene therapy, are disclosed.

Figure 2
Effect of Different Treatment Groups on Endothelial Cells *In Vitro*.



*- BAP vs BAP-p53 or BAP-TSP1, p<0.05

--BAP-p53 or BAP-TSP1 vs BAP-p53/BAP-TSP1, p<0.01

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European Patent
Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number

which under Rule 45 of the European Patent Convention EP 97 11 2154
shall be considered, for the purposes of subsequent
proceedings, as the European search report

| DOCUMENTS CONSIDERED TO BE RELEVANT | | | CLASSIFICATION OF THE APPLICATION (Int.Cl.6) |
|--|---|-----------------------|---|
| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | |
| A | WO 95 30330 A (DZAU VICTOR J) * page 21, line 37 - page 23, line 7 * --- | 1-15, 18-25 | C12N15/12 C12N15/24 A61K48/00 |
| A | WO 95 31559 A (ARCH DEV CORP ;DANA FARBER CANCER INST INC (US); OHNO TSUNEYA (US)) * page 3, line 11 - page 5, line 17 * * page 50, line 35 - page 52, line 33 * --- | 1-15, 18-25 | |
| A | WO 92 02240 A (REPLIGEN CORP) * page 5, line 9 - line 21 * --- | 1-15, 18-25 | |
| D,A | WEINSTAT-SASLOW ET AL: "TRANFECTION OF THROMBOSPONDIN 1 COMPLEMENTARY DNA INTO A HUMAN BREAST CARCINOMA CELL LINE REDUCES PRIMARY TUMOR GROWTH, METASTATIC POTENTIAL, AND ANGIOGENESIS" CANCER RESEARCH, vol. 54, 1994, pages 6504-6511, XP002048545 * page 6504 * * abstract * * page 6505, paragraph 2 * --- | 1-15, 18-25 -/- | TECHNICAL FIELDS SEARCHED (Int.Cl.6) A61K |
| INCOMPLETE SEARCH | | | |
| <p>The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.</p> <p>Claims searched completely:</p> <p>Claims searched incompletely:</p> <p>Claims not searched:</p> <p>Reason for the limitation of the search:</p> <p>see sheet C</p> | | | |
| Place of search | Date of completion of the search | Examiner | |
| THE HAGUE | 28 November 1997 | Sitch, W | |
| CATEGORY OF CITED DOCUMENTS | | | |
| <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons B : member of the same patent family, corresponding document</p> | | | |



PARTIAL EUROPEAN SEARCH REPORT

Application Number

EP 97 11 2154

| DOCUMENTS CONSIDERED TO BE RELEVANT | | CLASSIFICATION OF THE APPLICATION (Int.Cl.6) |
|-------------------------------------|--|---|
| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim |
| P,X | <p>WO 96 21416 A (VIAGENE INC)</p> <p>* page 4, line 17 - line 22 *</p> <p>* page 5, line 7 - line 16 *</p> <p>* page 9, line 27 - page 10, line 3 *</p> <p>* page 15, line 8 - page 17, line 5 *</p> <p>-----</p> | <p>1,2,5,6, 9-11,14, 20,21, 23,25</p> |
| | | TECHNICAL FIELDS SEARCHED (Int.Cl.6) |
| | | |
| | | |



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INCOMPLETE SEARCH
SHEET C

Application Number

EP 97 11 2154

Claim(s) searched completely:
1-15 18-25

Claim(s) not searched:
16 17

Reason for the limitation of the search:

Claims 16 and 17 are not readily comprehensible, and thus do not conform with the requirements of Article 84, EPC